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The Role of SDF1 /CXCR4 Signaling in Opioid-Induced Hypernociception

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LOYOLA UNIVERSITY CHICAGO

THE ROLE OF SDF1/CXCR4 SIGNALING IN OPIOID-INDUCED
HYPERNOCICEPTION

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN MOLECULAR PHARMACOLOGY & THERAPEUTICS

BY

NATALIE M. WILSON

CHICAGO, IL

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LIST OF ABBREVIATIONS

BBB	Blood Brain Barrier
BL	Baseline
BNB	Blood Nerve Barrier
CFA	Complete Freund's adjuvant
CGRP	Calcitonin Gene-Related Peptide
CNS	Central Nervous System
CRPS	Chronic Regional Pain Syndrome
CSF	Cerebral Spinal Fluid
DAMGO	[D-Ala ² , N-MePhe ⁴ , Gly-ol]-enkephalin
DOR	Delta Opioid Receptor
DPDPE	[D-Pen ^{2,5}]Enkephalin , [D-Pen ² ,D-Pen ⁵]Enkephalin
DRG	Dorsal Root Ganglion
ELISA	Enzyme-Linked Immunosorbant Assay
GPCR	G-protein coupled receptor
IL-1 β	Interleukin-1 β
IL-1R	Interleukin type 1 receptor
IL-8	Interleukin-8
IP-10	Interferon gamma-induced Protein 10 kDa

ID	Injection Day
IP	Intraperitoneal
IR	Immunoreactivity
KOR	Kappa Opioid Receptor
LPS	Lipopolysaccharide
M3G	Morphine-3-glucuronide
M6G	Morphine-6-glucuronide
MCP1	Monocyte Chemotactic Protein 1
MIP1	Macrophage Inflammatory Protein 1
MOR	Mu Opioid Receptor
NFAT	Nuclear Factor of Activated T cells
NSAIDS	Non-Steroidal Anti-Inflammatory Drugs
OIH	Opioid-Induced Hypernociception
PAG	Periaqueductal Grey
PID	Post Injection Day
PNS	Peripheral Nervous System
PWT	Paw Withdrawal Threshold
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
SDF1	Stromal Derived Factor 1
SGCs	Satellite Glial Cells
SPC	Spinal Cord
TLR4	Toll Like Receptor 4
TRPV1	Transient Receptor Potential Vanilloid 1

ABSTRACT

Opioids currently represent the best treatment option for severe and chronic pain conditions. Opioids while effective at controlling pain states also come with a number of side effects such as respiratory depression, urinary retention, dependence, tolerance, and opioid-induced hypernociception (OIH). OIH is a phenomenon in which opioids induce pain and this pain is often experienced at a site separate from the site of injury. Much research has been conducted investigating the mechanism of OIH, but the mechanism remains unsolved. One potential mechanism that has yet to be adequately explored is chemokines. Chemokines role in OIH is warranted given recent studies demonstrating the interaction between opioids and chemokines. Chemokines were originally thought to solely function in the immune system, but have recently been found to play a major role in the nervous system, as well as being implicated in a number of different pain models. Therefore, the purpose of these studies was to investigate a possible interaction between opioids and chemokines in the peripheral nervous system and the role this interaction plays in the development and maintenance of OIH. To do this, I tested for changes in expression of SDF1 and CXCR4 signaling in the dorsal root ganglion following repeated morphine administration. Secondly, I investigated if opioid or non-opioid signaling was involved in the development of OIH and which of these

receptor signaling cascades was responsible for changes in SDF1/CXCR4 signaling in the dorsal root ganglion. These studies employed the use of a number of different methods including animal behavior, in situ hybridization, immunocytochemistry, and calcium imaging.

It was found that SDF1/CXCR4 signaling was indeed increased in OIH and that these changes in SDF1/CXCR4 signaling occur following activation of the mu opioid receptor. Additionally, OIH appears to be induced by both opioid and non-opioid receptor signaling. These results suggest that opioids are inducing a neuroinflammatory process that can be detrimental at anatomical sites separate from an injury. Therefore, to improve the analgesic effectiveness of opioids these off target effects must be considered and new treatments that can bypass these effects should be explored.

CHAPTER ONE

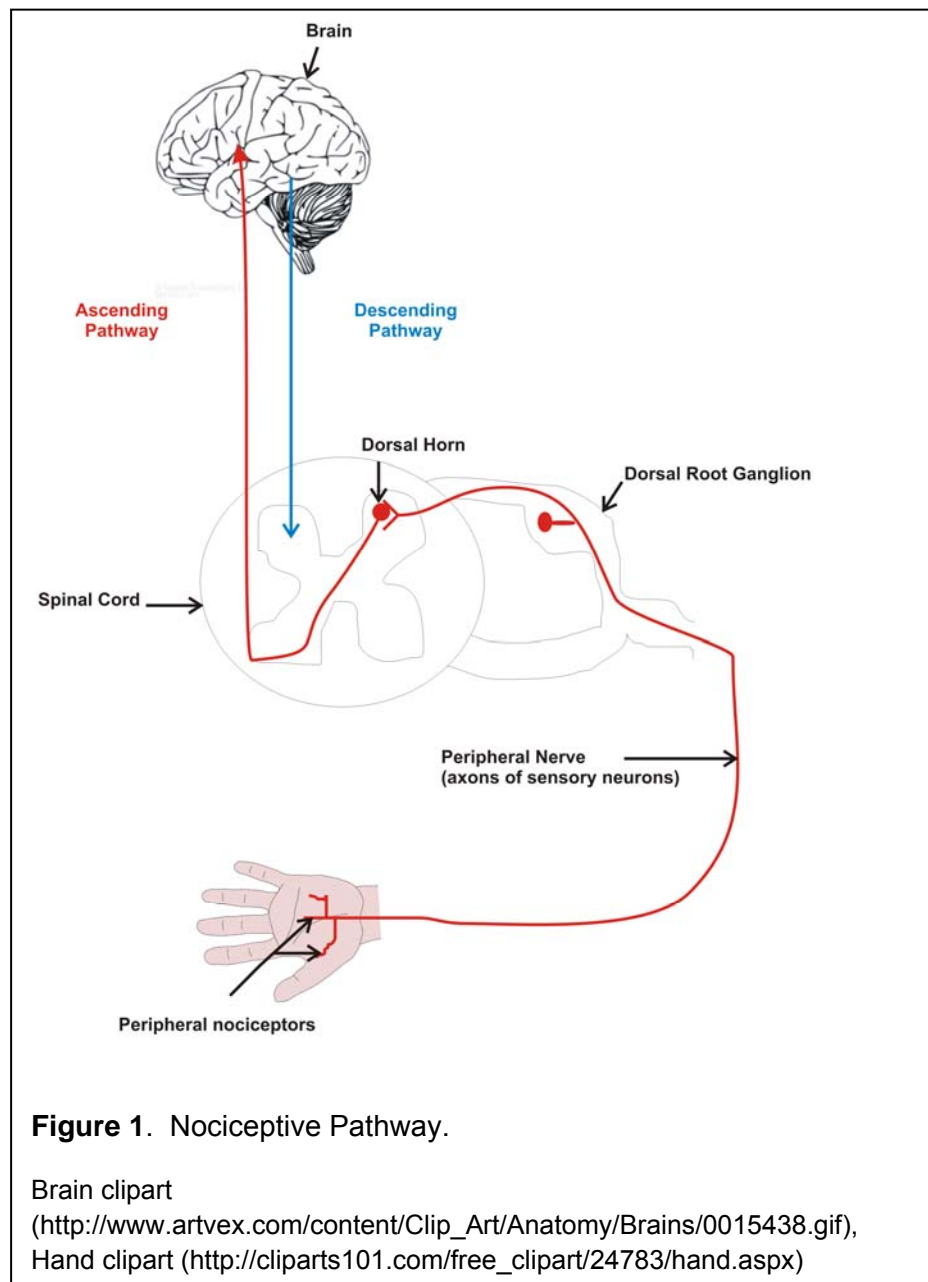
INTRODUCTION

Pain

The ability to experience pain has a beneficial role in humans and animals. For example, the ability to withdraw your hand following touching a hot object protects against tissue damage. Sherrington more than a century ago defined this type of stimulus as noxious, a stimulus with potential to damage tissue (Sherrington, 1906). Therefore, it is beneficial to be able to distinguish between noxious and non-noxious stimuli. Acute pain perception is essential for avoiding potential tissue damaging events which may hinder the healing process. In contrast, pain that persists beyond the wound healing period serves no beneficial role. In fact, this type of chronic pain contributes to deterioration in the quality of life. Chronic pain states are characterized using the following terms: hyperalgesia (increased sensitivity to painful stimuli), allodynia (non-noxious stimuli perceived as painful) and increased spontaneous pain. It is important to note that pain involves the perception of pain and is a subjective experience. Therefore, animal models often employ the term nociception which involves observations of neural, physiological, and behavioral changes.

Nociceptive Pathway

The nociceptive signal originates from stimuli activating nociceptors on primary afferent sensory neurons (**Fig.1**). Primary afferent sensory neurons transduce stimuli to an electrical signal that is then transmitted up the sensory neuron axon to the dorsal horn of the spinal cord, the site of the first synapse of the signal. Interneurons within the lamina I-II of the dorsal horn receive input from primary afferent neurons and are responsible for initiating the withdrawal reflex permitting fast reaction to potentially damaging stimuli. Primary afferent sensory neurons also synapse with second order neurons of lamina I-II within the dorsal horn of the spinal cord which transmit the signal to higher centers in the central nervous system, thalamus and cortex; transmission of this signal is referred as the ascending pathway. These supraspinal centers are responsible for the conscious and emotional experience of pain, are the site of conscious perception of pain in humans. Descending pathways originate in the cortex and project to the periaqueductal grey (PAG). Inhibitory neurons in PAG send axons to spinal cord dorsal horn and modulate incoming noxious signals from the periphery. The descending pathway is often referred to as the modulatory pathway because it synapses with dorsal horn and primary sensory neurons and can regulate the level of excitation of the nociceptive signal. Therefore, the nociceptive pathway has a built-in feedback system by which it can regulate the nociceptive experience.



Components of Dorsal Root Ganglia

Sensory Neurons

Primary afferent sensory neurons are pseudounipolar neurons. Sensory neurons have a single axon composed of two branches that extend from the cell body located in the dorsal root ganglion (DRG). One branch of the axon extends

to peripheral tissues such as skin, muscle, and viscera. This branch of the sensory neuron is responsible for sensing changes in temperature, touch, proprioception, and pain in the environment. The other branch of the sensory neuron extends into the dorsal horn of the spinal cord where it synapses and relays information from the periphery for further processing within the spinal and supraspinal level.

Sensory neurons within the DRG vary in size, degree of myelination, and conduction velocity. Large diameter, A α , and medium diameter, A β , sensory neurons are both fast conducting, myelinated axons that are in charge of relaying information about touch and proprioception. A subset of sensory neurons, nociceptive neurons, transmit sensory information about potentially damaging stimuli and thus are responsible for transmitting pain signals. Nociceptive sensory neurons are polymodal responding to mechanical, thermal, and chemical stimuli and are further divided into two types, A δ and C fibers. A δ are fast conducting, myelinated fibers and are often referred to as high-threshold mechanoreceptors (Burgess and Perl, 1967). A δ fibers are responsible for transmitting the initial stimuli of nociception. C fibers are slow conducting, unmyelinated fibers and transmits a less robust signal.

Sensory neurons have very unique and interesting characteristics. DRG neurons express receptors for neurotransmitter such as, glutamate, serotonin, ATP, bradykinin, Substance P, and GABA (Sato et al., 1993; Huettner, 1990; Lovinger and Weight, 1988; Todorovic and Anderson, 1990; Bean et al., 1990; Bouvier et al., 1991; Thayer et al., 1988; Spigelman and Puil, 1991; Aibara and

Akaike, 1991; Robertson, 1989). Cell somas of the DRG are devoid of any dendrites and synapses, but are capable of releasing neurotransmitters and inflammatory mediators such as CGRP, Substance P, and glutamate in a calcium- dependent manner (Hingtgen and Vasko, 1994; Mason et al., 1984; Vedder and Otten, 1991; Holz et al., 1988; Jeftinija and Jeftinija, 1990; Huang and Neher, 1996). DRG neurons are capable of sensing activity in neighboring neurons through transient depolarization (Liu et al., 1999; Utzschneider et al., 1992; Amir and Devor, 1996), changing their excitable state following nerve injury (Wall and Devor, 1983) and increasing the expression of sodium channels following inflammatory pain models (Tanaka et al., 1998; Gould et al., 1998). All of these changes would serve to change the excitability state of sensory neurons and thus change the state of the nociceptive pathway as a whole.

Satellite Glial Cells

The other important component of the DRG are satellite glial cells (SGCs) which surround each sensory neuron. Glial cell involvement in the nervous system has only recently begun to be explored. Glial cells were once thought to solely function as their name describes as the “glue” of the nervous system holding neurons in place. However, research conducted in recent decades has begun to demonstrate the importance of these cells in the physiology of the nervous system. Glial cells regulate the environment of the neurons they surround (Hansson and Ronnback, 2003; Reichenbach, 1991), as well as being important in the developing nervous system (Slezak and Pfrieder, 2003; Goldman, 2003). There is very little known about SGCs but they do express

receptors, transporters, ion channels, and ligands demonstrating their ability to signal and regulate the environment in the DRG (reviewed in (Hanani, 2005)). SGCs are unique in that they form a protective envelope/sheath around the sensory neurons of the DRG. Each sensory neuron is surrounded by several SGCs possibly serving to form a functional unit between each sensory neuron and its surrounding SGCs (Pannese, 1981; Hanani, 2005). Although the SGCs form an envelope around each sensory neuron, ions, neurotransmitters, and macromolecules are able to penetrate through the sheath allowing for cross-communication among sensory neurons in the DRG (Shinder and Devor, 1994). The SGC envelope can also serve to protect sensory neurons from toxic substances such as mercury and lead (Kumamoto et al., 1986; Schlaepfer, 1969). Therefore, it appears that the SGCs serve as the barrier for the sensory neuron's cell bodies within the DRG. Taken together these characteristics demonstrate the critical role SGCs have to regulate the environment of neurons.

Satellite glial cells are capable of increases in intracellular calcium levels in response to stimuli (England et al., 2001). Indeed, it appears that neuronal response to inflammatory mediators such as, bradykinin, is dependent on contact with SGCs (Heblich, 2001). Additionally, SGCs properties such as increased glial cell coupling and more depolarized resting membrane potential can be altered following chronic compression of the DRG (Zhang et al., 2009). Other interactions have been demonstrated between sensory neurons and SGCs through mechanisms involving the paracrine interaction of cytokine interleukin-1 β (IL-1 β) within the DRG. IL-1 β expression is increased in SGCs following

complete Freund's adjuvant (CFA) injection (Takeda et al., 2007). CFA treatment also induces increased firing frequency of sensory neurons (Takeda et al., 2007). Interleukin-1 receptor antagonist treatment inhibits spontaneous and mechanical stimulation-induced increased neuronal firing frequency in CFA treated animals (Takeda et al., 2008), suggesting IL-1 β is released from SGCs and acting on sensory neurons to induce the increased firing frequency. To this end, it appears that the satellite glial cells may play a crucial role in the signaling and modulating interactions that occur within the DRG.

Clinical Treatments for Acute and Chronic Pain

There are a number of treatment options for acute pain states, such as local anesthetics which temporarily prevent neuronal activity. Acute inflammatory pain states resulting from trauma or surgical procedures are often controlled with the use of non-steroidal anti-inflammatory drugs (NSAIDS). Where there is a lack of effective treatment options are in chronic clinical pain states. While a large percentage of the population suffer from chronic pain 40-50 percent of these individuals do not have adequate relief of their pain (Glajchen, 2001). The class of therapeutics prescribed varies for depending on severity level and time course of the pain state. Neuropathic pain states that result from injury to the nervous system, have limited treatment with only anti-convulsants and tri-cyclic anti-depressants being prescribed and have limited effectiveness. These drugs are chosen for their potential to decrease cell excitability. Chronic pain from inflammatory conditions is largely controlled by non-steroidal anti-inflammatory drugs (NSAIDS). In the case of severe pain states opioid analgesics are often

prescribed in an attempt to control pain because of their substantial analgesic ability. Opioids which target opioid receptors are often effective in controlling pain; however, they come with a number of side effects, such as respiratory depression, urinary retention, dependence, tolerance and finally opioid-induced hypernociception, which will be explained in more detail in later sections.

G Protein Coupled Receptors

G-protein coupled receptors (GPCRs) are a large family of receptors that represent the target receptor for the majority of physiologically relevant ligands. GPCRs are seven transmembrane receptors that are coupled to heterotrimeric G proteins. Ligand binding to the receptor initiates a conformational change allowing for the activation of the G protein. The heterotrimeric G protein is composed of α , β , and γ subunits. G proteins are classified by their α subunit and each α subunit initiates a different cascade of events. G_s which activates adenylyl cyclase, G_i which inhibits adenylyl cyclase, and G_o and G_q which activate phospholipase C which then activates IP_3 and diacylglycerol production.

GPCR signaling can be regulated through the desensitization process. Following ligand binding to the GPCR, the GPCR is phosphorylated by G-protein-linked receptor kinases (GRKs). GRK phosphorylation of the receptor allows for arrestin binding to the receptor. Arrestin binding to the receptor sterically hinders the G-protein from binding to the receptor, thereby uncoupling the G-protein from the receptor and preventing further receptor activation. Arrestin binding to the receptor can also serve to initiate receptor internalization, further preventing ligand and receptor signaling events.

Opioid Receptors

Opioid receptors are a family of seven transmembrane GPCRs that consist of three types, μ (MOR), δ (DOR), and κ (Kappa). Opioid receptors are thought to be G_i coupled receptors. Some studies have suggested that opioid receptors are also G_s coupled under both naïve and morphine treated conditions (Chakrabarti and Gintzler, 2007; Chakrabarti et al., 2005; Chakrabarti et al., 1998). All three opioid receptors were identified and cloned in the early 1990s (Evans et al., 1992; Chen et al., 1993; Meng et al., 1993). MOR, DOR, and KOR have homologous sequences, with the highest conserved sequences in the transmembrane and intracellular regions (63-76%). However, the extracellular regions, typically responsible for ligand binding are less conserved (34-30%) (Minami and Satoh, 1995).

Opioid receptors are expressed throughout the nervous system both centrally and peripherally. In particular opioid receptor expression correlates strongly with the regions important in the nociceptive pathway. Opioid receptors are located in lamina I-III of the spinal cord and at the supraspinal level with the greatest expression in the striatum, amygdala, thalamus, and PAG of the central nervous system (Kuhar et al., 1973; Pert et al., 1976; Lamotte et al., 1976; Atweh and Kuhar, 1977; Fields et al., 1980; Ninkovic et al., 1982). Peripheral distribution of the opioid receptors extends to cutaneous skin (Stein et al., 1990; Pare et al., 2001) and all opioid receptors are expressed on small, medium, and large diameter DRG neurons (Fields et al., 1980; Mansour et al., 1994; Minami et al., 1995; Buzas and Cox, 1997; Chen et al., 1997; Coggeshall et al., 1997;

Zhang et al., 1998; Wang and Wessendorf, 2001; Silbert et al., 2003; Rau et al., 2005; Gendron et al., 2006).

Opioid Agonists and Antagonists

Each opioid receptor has both endogenous and exogenous ligands for which they have varying binding affinities, outlined in the table below.

Exogenous natural and synthetic opioids are often employed for pain treatment.

The analgesic mechanism of action of opioid agonists has long been believed to be through MOR. This can be witnessed in the prototypical opioid analgesic, morphine, which has its greatest affinity for the MOR (14 nM) followed by lesser affinity for KOR (538 nM) and DOR (>1000 nM) (Raynor et al., 1994) (**Table 1**).

Selective opioid agonists and antagonists exist for each of the opioid receptors and are often employed as pharmacological tools for identifying the role of each receptor in signaling events. For example, DAMGO, a selective exogenous MOR agonist, has a 1000 fold greater affinity for the MOR over DOR and KOR (Schiller et al., 1989; Schiller et al., 1990; Raynor et al., 1994) (**Table 1**). Non-selective opioid antagonists naloxone and naltrexone have similar affinities for the opioid receptors (**Table 1**), but differ by naltrexone having a longer half life than naloxone (Verebey et al., 1976).

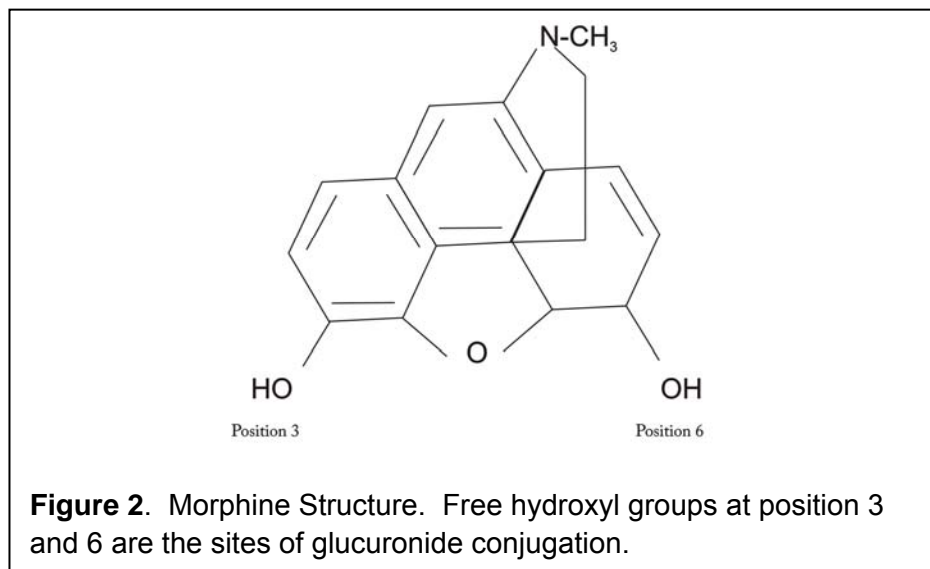
Receptor Type	Agonists	Antagonists
μ (MOR)	DAMGO Morphine	CTAP Naloxone Naltrexone
K (KOR)	U-50,488 Morphine	Nor-BNI Naloxone Naltrexone
δ (DOR)	DPDPE Deltorphin Morphine	Naltrindole Naloxone Naltrexone

Table 1. Opioid agonists and antagonists for each of the opioid receptors.

Peripheral versus Central Opioid Analgesic Actions

Opioid receptors are expressed throughout the central and peripheral nervous system as outlined previously; therefore the analgesic actions of opioid agonists could be acting through both. Classically, opioid agonists have been thought to carry out their analgesic ability through actions in the spinal and supraspinal level, such as the PAG. However, the ability of opioids to carry out their analgesic ability through acting on peripheral sites is possible because opioid receptors are located on sensory neurons (Fields et al., 1980; Mansour et al., 1994; Minami et al., 1995; Buzas and Cox, 1997; Chen et al., 1997; Coggeshall et al., 1997; Zhang et al., 1998; Wang and Wessendorf, 2001; Silbert et al., 2003; Rau et al., 2005; Gendron et al., 2006) and co-localize with known nociceptive substances, CGRP and Substance P (Dado et al., 1993; Wenk and Honda, 1999; Minami et al., 1995). Additional evidence for opioids peripheral analgesic ability is provided by studies demonstrating activation of mu opioid

receptors on sensory neurons induces: (1) a decrease in spontaneous activity (Russell et al., 1987), (2) decrease in calcium currents (Werz and Macdonald, 1982; Borgland et al., 2001), (3) decrease in non-selective cation currents (Ingram and Williams, 1994), and (4) inhibits activation through prostaglandin (Gold et al., 1996), Transient Receptor Potential Vanilloid 1 (TRPV1) (Endres-Becker et al., 2007), and purinergic receptors (Chizhnikov et al., 2005). Several studies support peripheral action of opioid analgesics through the use of peripherally restricted opioid agonists and antagonists (Stein et al., 1991; Barber et al., 1994; Aley et al., 1995; Zhou et al., 1998; Likar et al., 1999; Machelska et al., 1999; Koppert et al., 1999; Pertovaara and Wei, 2001; Dionne et al., 2001; Reichert et al., 2001; Shannon and Lutz, 2002; Junger et al., 2002; Furst et al., 2005; Labuz et al., 2007; Mousa et al., 2007). In fact, it has been demonstrated that 50-80% of the analgesic ability of systemically administered opioids is carried out through their actions on peripheral targets (Reichert et al., 2001; Shannon and Lutz, 2002; Furst et al., 2005; Labuz et al., 2007).



Morphine Metabolism

Morphine undergoes first pass metabolism and is mainly metabolized in the liver by the process of glucuronidation. Glucuronidation is a conjugation process carried out by the hepatic enzyme UDP-glucuronyl transferase, UGT2B7. Glucuronide conjugation can occur at both the 3 and 6 position free hydroxyl sites on the morphine structure (**Fig. 2**) resulting in the formation of the two major metabolites of morphine, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) (reviewed in (Coller et al., 2009)). M6G shares similar affinities at MOR, DOR, and KOR as its parent compound, morphine (Pasternak et al., 1987) and 9-10% of morphine is converted to M6G (Osborne et al., 1990; Hasselstrom and Sawe, 1993). Because M6G has similar affinity for the MOR its analgesic ability is retained (Pasternak et al., 1987; Penson et al., 2000). M3G is the major metabolite of morphine with 44-55% of morphine being converted to M3G (Osborne et al., 1990; Hasselstrom and Sawe, 1993).

Although M3G is a major metabolite of morphine it has very little affinity for the MOR, DOR, and KOR opioid receptors (Pasternak et al., 1987) with a greater than 100 fold less affinity for the MOR compared to morphine (Skarke et al., 2005). M3G's loss of affinity for the opioid receptors is attributed to the lack of a free 3-hydroxyl group that is needed for strong affinity to opioid receptor binding (Pert and Snyder, 1973). Thus, because M3G has limited affinity for the opioid receptors it does not possess analgesic abilities (Pasternak et al., 1987; Ekblom et al., 1993). In fact, M3G has been proposed to be responsible for the negative side effects following morphine administration. Increased plasma and cerebral spinal fluid levels of M3G correlate with decreases in analgesic activity of morphine (Baker and Ratka, 2002; Barjavel et al., 1995). M3G has also been shown to antagonize the analgesic effects of morphine when co-administered (Smith et al., 1990) and can be neuroexcitatory and lead to nociceptive behavior (Bartlett et al., 1994; Labella et al., 1979; Yaksh et al., 1986; Woolf, 1981; Lewis et al., 2010). Therefore, it is believed that the morphine metabolite, M3G, could be responsible for one of the negative side effects of morphine such as, opioid-induced hypernociception.

Opioid-Induced Hypernociception

Opioids, such as morphine, currently represent the best option for the management of moderate to severe trauma-induced, perioperative and cancer pain. Opioid compounds are also increasingly being used for chronic, non-cancer chronic pathological pain. However, prolonged administration of opiates is associated with significant problems including the development of

antinociceptive tolerance, wherein higher doses of the drug are required over time to elicit the same amount of analgesia. These higher doses are also thought to increase pain sensitivity, a concept known as opioid-induced hypernociception (OIH). This increased pain is usually experienced at a location separate from the original site of injury ([Ossipov et al., 2004](#)).

OIH has been observed both clinically ([Angst et al., 2003](#); [Arner et al., 1988](#); [Singla et al., 2007](#)) and experimentally ([Laulin et al., 1999](#); [Woolf, 1981](#)). Many explanations for this phenomenon have been suggested. For example, OIH was once believed to occur as a result of “mini withdrawals”, however OIH still occurs when opiates are constantly infused ([Vanderah et al., 2000](#); [Vanderah et al., 2001](#)). Some investigators will even go so far as to suggest that OIH is actually a form of antinociceptive tolerance, in which patients require a greater opiate dose in order to receive the same analgesic effect ([Guignard et al., 2000](#); [Luginbuhl et al., 2003](#)). Yet another explanation is that the hyperalgesic response to morphine is caused by a compensatory response to the inhibition produced by activation of the mu opioid receptor (MOR), causing a hyperactivity of the system ([Gutstein, 1996](#)). In fact, higher doses are suggested to precipitate this effect largely because the hyperactive state becomes more dominant ([Colpaert, 2002](#)).

Release of the neurotransmitter, glutamate, has also been implicated as an entity involved in OIH. To this end, glutamate antagonism in the dorsal horn of the spinal cord has been somewhat effective in temporarily reversing OIH ([Celerier et al., 2000](#); [Laulin et al., 1998](#)). The involvement of glutamate

receptors is not surprising because the long lasting effects that are witnessed in OIH would require neural plasticity, changes that likely require glutamate receptors. However, the ability of glutamate blockade to effectively treat OIH is questioned. This is because the neural plastic changes that are occurring are present in two parts; i) the sensitivity of the glutamate receptor, and ii) the perceived decreased responsiveness of the MOR. Blockade of the glutamate receptor would transiently reverse the nociceptive behavior, however it does not address the changes that have occurred in the MOR bearing cells (Mao et al., 1995). Despite a considerable amount of work on the topic little is known about the underlying mechanism.

Much of the current studies on OIH have focused on the CNS, but the mechanism of OIH largely remains unsolved. Given the peripheral actions of opioids outlined previously, peripheral opioid mechanisms role in the induction of OIH seems likely, but have largely been unexplored. Another potential mechanism that has yet to be explored in OIH is the role of chemokines which in recent years has been connected to many pain models.

Interaction between Opioids and Chemokines

Chemokines and opioids are often co-expressed in various tissues and cells. The first interaction noted was in the immune system. Macrophages and T-lymphocytes express opioid receptors (Chuang et al., 1994; Chuang et al., 1995; Wick et al., 1996; Wybran et al., 1979) and these cell types also express chemokine receptors. Because opioid agonists are often employed to control inflammatory pain states, researchers sought to determine any effect that opioid

treatment would have on immune system function. Chemotaxis achieved by the chemoattractant gradient of chemokines is utilized by immune cells in order to effectively move to their site of action. Therefore chemotaxis is often employed as a functional test for cells of the immune system. Pretreatment with opioid agonists reduces the chemotactic response to chemokines (Chen et al., 2004; Grimm et al., 1998; Choi et al., 1999; Rogers et al., 2000; Miyagi et al., 2000). This reduction in chemotaxis is attributed to the heterologous cross-desensitization between opioids and chemokines (Chen et al., 2004; Grimm et al., 1998; Szabo et al., 2002). Desensitization is utilized by GPCRs to regulate the number of receptors that are available for ligand binding and receptor activation. Desensitization occurs by phosphorylation of the GPCR following ligand binding and receptor activation, this phosphorylation sterically hinders the G-protein from binding to the receptor, thereby preventing downstream signaling events from occurring following ligand binding to the receptor. All GPCRs share this mechanism therefore this process can occur through two means: 1) a receptor becoming phosphorylated following its own activation, homologous desensitization and 2) one GPCR in a cell being activated and causing phosphorylation of other GPCRs within the same cell, heterologous desensitization.

Another interaction between opioids and chemokines in the immune system was discovered when a correlation was found among opioid abusers and their susceptibility to HIV infection (Donahoe and Vlahov, 1998). This correlation was long attributed to lifestyle choices of addicts, unprotected sex and sharing

needles. However other explanations began to be considered when studies demonstrated that morphine administration elevates HIV replication (Peterson et al., 1990; Peterson et al., 1994; Chuang et al., 1993). Follow-up studies discovered that this correlation could be attributed to the ability of opioid agonists such as, morphine and DAMGO, to increase the expression of the chemokine HIV co-receptors, CXCR4 and CCR5, on monocytes and lymphoblasts (Steele et al., 2003; Miyagi et al., 2000). Increased chemokine expression on immune cells essentially acts to increase the probability for viral entry/infection into cells.

As chemokines role in systems other than the immune system became evident, possible interactions between opioids and chemokines in the nervous system began to be explored. Several *in vitro* studies demonstrated that chronic morphine treatment led to an upregulation of CCL2 in human neurons (Rock et al., 2006), CCR2 and CCR5 in human astrocytes (Mahajan et al., 2005), and MCP1 in astrocytes (El-Hage et al., 2006). These studies repeated what had already been demonstrated in the immune system that opioid treatment leads to changes in chemokine/receptor expression. Functional interactions within the nervous system were also observed. Similar to the effect on chemotaxis in the immune system, heterologous desensitization also occurs in the nervous system. The Rogers and Adler group demonstrated chemokine injections into the periaqueductal grey (PAG) leads to a decrease in the anti-nociceptive effects of opioids (Szabo et al., 2002; Chen et al., 2007a; b). The chemokine induced decrease in opioid anti-nociception was short, lasting only 2 hours following chemokine administration (Szabo et al., 2002). These short term, acute effects

were attributed to a heterologous desensitization mechanism, since changes in expression pattern are unlikely to occur in the utilized time frame. Follow-up studies demonstrated intra-PAG administration of chemokines RANTES and SDF1 prior to systemic morphine injection is ineffective in decreasing the analgesic effect of morphine (Adler et al., 2005). Chemokines ineffectiveness to completely block the analgesic effects of systemically administered morphine can be attributed to morphine acting on opioid receptors in both the central and peripheral nervous system to carry out its analgesic effect, as outlined previously. Heterologous desensitization between chemokines and opioids has also been demonstrated on *in vitro* culture conditions of the DRG, in which chemokine pretreatment led to a decrease in DAMGO induced calcium influx and decreased MOR present on the plasma membrane (Zhang et al., 2004a). Current studies have provided evidence of an interaction between chemokines and opioids following acute administration and have attributed this acute interaction largely to heterologous desensitization. However, the presence of chronic long lasting interactions among these two signaling systems has yet to be demonstrated.

Chemokines/Receptors

Chemokines (chemotactic cytokines) are a family of small proteins (10-14 kDa) traditionally thought to be involved in leukocyte trafficking under normal physiological and pathological conditions, as well as signaling in the developing and injured adult nervous system. Chemokines are typically classified by the presence of a cysteine motif in the N-terminal region of the protein (Zlotnik and Yoshie, 2000). Initial characterization of chemokines divided the family into α -

and β chemokines. In α chemokines, one amino acid separates the first two cysteine residues (cysteine-X amino acid-cysteine or CXC), whereas in β -chemokines, the first two cysteine residues are adjacent to each other (cysteine–cysteine, or CC). Two additional classes were added for the chemokines, lymphotactin (single cysteine, XC) and fractalkine (first two cysteines are separated by three amino acids, CX3C). The chemokine nomenclature herein utilizes both the original ligand name and the systematic name. The systematic name uses XC, CC, CXC and CX3C, indicating the class to which the chemokine belongs, followed by the letter “L” (for ligand) and then a number. The numbering system corresponds to that already in use to designate the genes encoding each chemokine. All chemokines exert their biological effects through the activation of an extended family of seven transmembrane G-protein coupled receptors (GPCRs). Nineteen chemokine receptors have been cloned including six CXC receptors (CXCR1-7), 10 CC receptors (from CCR1-10) and two single receptors each for lymphotactin (XCR1) and fractalkine (CX3CR1). Chemokine receptors are notoriously promiscuous, i.e. single chemokines can activate several different chemokine receptors. There are, however, instances when a chemokine receptor is uniquely activated by a single chemokine.

Stromal derived factor 1(SDF1) and CXCR4

Stromal derived factor 1 (SDF1) was first identified in 1993 from murine bone marrow, hence the name (Li and Ransohoff, 2008; Tashiro et al., 1993). SDF1 was given the new name of CXCL12 in accordance with the new systematic naming system. SDF1 is highly conserved between mice and

humans differing by only one amino acid, is widely expressed throughout the body, and exhibits a broad range of actions affecting stromal cell migration, leukocyte chemotaxis, vascularization of multiple organ systems, metastatic tumor formation, neural development and chronic pain (reviewed in (Miller et al., 2008; White et al., 2007)). The molecular structure of SDF1 exhibits an amino acid sequence that contains four cysteine residues conserved by most CXC chemokines with the N-terminus of SDF1 particularly important for activity. The monomer form of SDF1 is known to produce internalization of its receptor, CXCR4, and intracellular calcium mobilization. Recent studies using nuclear magnetic resonance structure analysis of the SDF1: CXCR4-N-domain complex have also determined that the structural basis of the recognition of receptor residues by the chemokine is indicative of a constitutively active dimeric form of SDF1. Importantly, this dimeric form serves only to activate intracellular calcium mobilization (Veldkamp et al., 2008). The differential effects on CXCR4-bearing cells by either the monomeric or dimeric forms reveal the latter to be a potent partial agonist (Veldkamp et al., 2008).

The receptor for SDF1 was identified from the orphan GPCR, LESTR/fusin whose name was later changed to CXCR4 reflecting its ability to bind and respond to SDF1 (Bleul et al., 1996; Oberlin et al., 1996). CXCR4 was also the first identified co-receptor for human immunodeficiency virus (HIV-1) (Feng et al., 1996). CXCR4 is a G α i coupled GPCR capable of inducing calcium influxes (Boutet et al., 2001; Gillard et al., 2002). The importance of SDF1/CXCR signaling in nervous system development is continuing to be explored.

SDF1/CXCR4 signaling importance in the development of the DRG was demonstrated in CXCR4 $-/-$ embryonic mice, which have small malformed DRGs (Belmadani et al., 2005).

Until recently, CXCR4 was known to be the only receptor for SDF1. This idea was challenged when the chemokine receptor, CXCR7, was shown to bind SDF1 (Balabanian et al., 2005). Initially described as a scavenger receptor (Boldajipour et al., 2008; Dambly-Chaudiere et al., 2007), more recent interactions describe CXCR7 as possibly moderating the response of CXCR4 to SDF1 by internalizing the ligand (Zabel et al., 2009). Although CXCR7 does not elicit activation of G-protein signaling pathways, it does activate MAP kinases through β -arrestin (Rajagopal et al., 2010).

AMD3100, a CXCR4 antagonist

AMD3100 is a bicyclam antagonist that was originally developed for the treatment of HIV infection. Its mechanism of action for HIV treatment being to block viral binding to the HIV co-receptor CXCR4 and therefore limiting viral entry (Schols et al., 1997; Donzella et al., 1998). AMD3100 selectively blocks viral entry of R4 not R5 viral strains (Schols et al., 1997). Additional functional studies of AMD3100 showed that AMD3100 blocked CXCR4 antibody labeling and inhibited SDF1-induced calcium responses (Schols et al., 1997; Donzella et al., 1998). AMD3100 has a 1000 fold greater affinity for CXCR4 over other chemokine receptors, CXCR1-3, CCR1-9 (Hatse et al., 2002). An alternative chemokine receptor target for AMD3100 was demonstrated at CXCR7, where AMD3100 functions as an allosteric agonist (Kalatskaya et al., 2009). However,

ongoing studies have demonstrated CXCR7 signals in a non-G-protein manner (Rajagopal et al., 2010). Therefore AMD3100 agonist actions at CXCR7 should be re-examined taking into account non-G protein signaling.

Chemokines and Pain

Immune and non-immune cells associated with the injury response release pro-inflammatory mediators such as prostaglandins, histamine, serotonin, protons, bradykinin, nerve growth factor, and pro-inflammatory cytokines that can sensitize primary afferent neurons and contribute to pain hypersensitivity. There is also adequate evidence demonstrating that like other inflammatory mediators, chemokines elicit hypernociception. For example, Oh and colleagues (2001) demonstrated that a single injection into the un-inflamed adult rat hind paw of SDF1/CXCL12, Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES/CCL5) or macrophage inflammatory protein 1 (MIP1/CCL3) produces dose-dependent tactile allodynia. These behavioral experiments in combination with accompanying RT-PCR, calcium imaging studies and immunohistochemistry confirmed the presence and functionality of the respective chemokine receptors, CXCR4, CCR5 and CCR4 in rodent dorsal root ganglion (DRG) sensory neurons (Oh et al., 2001). Similar behavioral effects were observed following the introduction of interleukin-8 (IL-8 CXCL8) (Cunha et al., 1991) and intrathecal introduction of fractalkine (CX3CL1) (Milligan et al., 2005). Studies conducted through intra- PAG injections of RANTES/CCL5 demonstrated a similar dose dependent decrease in rat tail flick latency (Benamar et al., 2008a).

Perhaps, the chemokine/receptor pairing that has been studied the most extensively for its role in nociceptive behavior is MCP1/CCR2. The importance of MCP1/CCR2 in neuropathic pain states was first demonstrated in CCR2 knockout mice. Testing of acute pain behavior in CCR2 knockout mice does not differ from wild type mice. Following partial ligation of the sciatic nerve, a model known to induce hypernociception, CCR2 knockout mice failed to display mechanical hyperalgesia (Abbadie et al., 2003), while overexpression of glial MCP1 by transgenic mice produced enhanced nociceptive responses (Menetski et al., 2007). Additional confirmation of a *de novo* role for MCP1/CCR2 signaling in injured neurons was observed following chronic compression of the dorsal root ganglia (a model of spinal stenosis). In this investigation, the injury produced neuronal upregulation of both MCP1 and CCR2 in the DRG while exogenous administration of MCP1/CCL2 produced a depolarized resting membrane potential and increased firing in the neuronal cell bodies (White et al., 2005). Subsequent studies demonstrated that sensory neurons following peripheral nerve injury exhibit chronic upregulation of functional MCP1/CCR2 signaling, and a CCR2 selective receptor antagonist could reverse hypernociceptive behavior in the injured animal (Bhangoo et al., 2007a). Further investigations into the excitatory effects of MCP1/CCR2 signaling in sensory neurons have revealed that i) regulation of the CCR2 chemokine receptor expression in neurons is activity-dependent on the signal transcription factor, nuclear factor in activated T cells (NFAT) (Jung and Miller, 2008) and ii) MCP1 activates a non-cation selective voltage-independent, depolarizing current and inhibited a voltage

dependent outward current (Sun et al., 2006). Moreover, MCP1 protein expression by DRG neurons following nerve injury is colocalized with calcitonin gene-related peptide in large dense core vesicles and release of MCP1 vesicles could be induced from the soma by depolarization in a Ca^{2+} -dependent manner (Jung et al., 2008). The role of MCP1/CCR2 signaling is not limited to the DRG soma. Zhang and De Koninck (2006) recently demonstrated that MCP1/CCL2 is also present in central afferent fibers in the spinal cord. Electrical activity due to peripheral nerve injury may serve to stimulate central afferent release of MCP1/CCL2 into the spinal cord dorsal horn activating CCR2 bearing glial cells or neurons (Abbadie et al., 2003; Zhang et al., 2007; Zhang and De Koninck, 2006).

A less extensively studied chemokine and receptor pairing for the involvement in nociception is SDF1/CXCR4. SDF1 application to sensory neuron cultures induces excitation and substance P release (Oh et al., 2001). This same study demonstrated that hindpaw injection of SDF1 decreases paw withdrawal threshold (Oh et al., 2001). Additionally, SDF1 injected into the PAG decreases the analgesic ability of opioid and cannabinoid agonists (Szabo et al., 2002; Benamar et al., 2008b). SDF1/CXCR4 signaling has been shown to be central to chronic constriction injury (CCI) of the sciatic nerve (Dubovy et al., 2010) and the nucleoside reverse transcriptase inhibitor, 2',3'-dideoxycytidine (ddC)-induced tactile nociceptive behavior (Bhangoo et al., 2007b).

Functional expression of chemokine/receptors in the damaged nervous system may both participate in the etiology and symptomology of diverse

pathological pain states. To date, the evidence in animal models includes the upregulation of chemokine/receptors in partial ligation of the sciatic nerve (Abbadie et al., 2003; Tanaka et al., 2004; Lindia et al., 2005; Zhang et al., 2007), chronic constriction injury of the sciatic nerve (Milligan et al., 2004; Zhang and De Koninck, 2006; Kleinschnitz et al., 2005; Dubovy et al., 2010), chronic compression of the L4, L5 DRG (CCD; a rodent model of spinal stenosis) (White et al., 2005; Sun et al., 2006), spinal cord contusion (Knerlich-Lukoschus et al., 2008), chemically-induced focal nerve demyelination (Bhangoo et al., 2007a; Jung et al., 2007), bone cancer pain (Vit et al., 2006), zymosan or adjuvant-induced inflammatory pain (Verge et al., 2004; Xie et al., 2006; Jeon et al., 2008; Sun et al., 2007) and the chemotoxic effects of some anti-HIV therapeutics (Bhangoo et al., 2007b). Despite the potential importance of these factors for clinical pain syndromes, only a few studies have been designed to investigate the presence of altered levels of chemokines. These include the measurement of chemokine levels in prostatic secretion from individuals diagnosed with chronic pelvic pain syndrome (Desireddi et al., 2008), herniated lumbar intravertebral disc specimens (Ahn, 2002) and the cerebral spinal fluid taken (CSF) from individuals diagnosed with chronic regional pain syndrome (CRPS) (Uceyler et al., 2007; Alexander et al., 2007; Alexander et al., 2005). Although these studies did not reveal a specific molecule that could serve as a diagnostic marker of a chronic pain syndrome, it was notable that CSF from patients afflicted with CRPS did reveal a common pattern of elevated cytokines and chemokines in 11 of 22 individuals tested (Alexander et al., 2007).

Goals

In summary, the information outlined in the preceding sections has demonstrated the need for a greater understanding of the mechanisms of opioid induced hypernociception. The mounting evidence of the interaction between opioids and chemokines in both the immune and nervous system, together with numerous studies showing the role that chemokines play in nociceptive models led me to pursue studies addressing the role that chemokines play in opioid induced hypernociception. Furthermore, the peripheral nervous system, in particular the DRG, has been overlooked in studies examining OIH. Providing evidence about the interaction of opioids and chemokines in sensory neurons will prove beneficial in developing new therapeutic options for opioid analgesics or treatment options targeted at chemokine signaling.

In the first part, Chapter 2, of this project I set out to demonstrate an upregulation of SDF1/CXCR4 signaling in the dorsal root ganglion through the use of immunocytochemistry and in situ hybridization experiments in the DRG. Calcium imaging studies were employed to test the presence of functional chemokine receptors. Finally, CXCR4 antagonism was used to demonstrate SDF1/CXCR4 signaling as a central component of OIH. The second part of this project, Chapter 3, my focus was to determine whether opioid or non-opioid receptor signaling was responsible for changes in SDF1/CXCR4 signaling witnessed in morphine-induced hypernociception. I utilized a number of pharmacological compounds to dissect the opioid and non-opioid receptor signaling components of OIH. These studies also employed the use of

immunocytochemistry techniques to examine changes in the expression of CXCR4 in the rodent DRG. Calcium imaging studies were used to test for the presence of functional chemokine receptors. CXCR4 antagonism was again utilized to determine the involvement of SDF1/CXCR4 signaling in any behavior induced by each of the pharmacological agents that were employed. The results of these experiments provide evidence of SDF1/CXCR4 signaling in opioid induced hypernociception and provide further details about the mechanisms behind opioid induced hypernociception.

CHAPTER TWO

THE ROLE OF SDF1/CXCR4 SIGNALING IN MORPHINE-INDUCED HYPERNOCICEPTION

Overview

Morphine and related compounds are the first line of therapy in the treatment of moderate to severe pain. Over time, individuals taking opioids can develop an increasing sensitivity to noxious stimuli, even evolving into a painful response to previously non-noxious stimuli (opioid-induced hypernociception; OIH). The mechanism underlying OIH is not well understood although complex intracellular neural mechanisms, including opioid receptor desensitization and down-regulation, are believed to be major mechanisms underlying OIH. However, OIH may also be associated with changes in gene expression. A growing body of evidence suggests that cellular exposure to mu agonists upregulate chemokines/receptors and recent work from our lab implicates chemokine upregulation in a variety of neuropathic pain behaviors. Here we characterized the degree to which chemokines/receptors signaling is increased in primary afferent neurons of the dorsal root ganglion (DRG) following chronic morphine sulphate treatment and correlated these changes with tactile hypernociceptive behavior in rodents. We demonstrate that mRNA expression of the chemokine,

stromal-derived factor 1 (SDF1/CXCL12) is upregulated following morphine treatment in sensory neurons of the rat. The release of SDF1 was found to be constitutive when compared with the activity dependent release of the C-C chemokine, monocyte chemoattractant protein 1 (MCP1/CCL2) in a line of *F-11* neuroblastoma-sensory neuron hybrid cells. We further determined that there is pronounced CXCR4 expression in satellite glial cells, and following morphine treatment, increased functional CXCR4 expression in sensory neurons of the DRG. Moreover, intraperitoneal administration of the selective CXCR4 antagonist, AMD3100, completely reversed OIH in the rat. Taken together; the data suggest that opioid-induced SDF1/CXCR4 signaling is central to the development of long lasting OIH and that receptor antagonists represent a promising novel approach to the management of the side effects associated with the use of opioids for chronic pain management.

Background

Opioids such as morphine currently represent the best option for the management of moderate to severe trauma induced, perioperative and cancer pain. Opioid compounds are also increasingly being used for non cancer associated chronic pathological pain. However, prolonged administration of opioids is associated with significant problems including the development of anti-nociceptive tolerance, wherein higher doses of the drug are required over time to elicit the same degree of analgesia. Repeated administration of higher doses of morphine or fentanyl also results in increasing pain sensitivity, a syndrome

clinically known as opioid-induced hypernociception (OIH) (Angst et al., 2003; Arner et al., 1988; Singla et al., 2007). This increased pain is usually experienced at different locations from the original site of injury (Ossipov et al., 2004).

While it is thought that opioids modulate tactile hypernociception solely by acting at neuronal opioid receptors, administration of chronic morphine is also known to induce a rapid increase in the expression of proinflammatory cytokines such as $\text{TNF}\alpha$, $\text{IL}1\beta$ and IL-6 in a number of cell types within the nervous system (Johnston et al., 2004). These proinflammatory cytokines are powerful pain enhancing proteins that may, in turn, suppress acute opioid analgesia and contribute to the apparent loss of opioid analgesia upon repeated opioid administration (“tolerance”) (Hutchinson et al., 2008). The family of pro-nociceptive cytokines includes chemotactic cytokines (chemokines). Proalgesic effects of chemokines have been implicated in both acute and chronic tactile hypernociceptive behavior (Abbadie et al., 2003; Bhangoo et al., 2007a; Bhangoo et al., 2007b; Johnston et al., 2004; Jung et al., 2009; Menetski et al., 2007; Milligan et al., 2004; Oh et al., 2001; Wang et al., 2008; White et al., 2005; Xie et al., 2006). However, the degree to which chronic morphine treatment alters gene expression of chemokines and their receptors, and whether this contributes to syndromes such as OIH is unknown.

Effects of opioids on chemokine receptor expression are potentially important determinants of HIV-1 infection rates among intravenous drug users, as the chemokine receptors CCR5 and CXCR4 are co-receptors for the HIV-1 virus coat protein, gp120. To this end a number of studies using chronic morphine or the

selective μ opioid agonist, [D-Ala², N-MePhe⁴, Gly-ol]-enkephalin (DAMGO) produce increased expression of monocyte chemoattractant protein-1 (MCP1/CCL2), regulated upon activation normal T-cell expressed and secreted (RANTES/CCL5), and their respective receptors, CCR2 and CCR5, in astrocytes and neurons via largely unknown mechanisms (Avdoshina et al., 2010; Mahajan et al., 2005; Rock et al., 2006). A similar study demonstrated that DAMGO substantially increased the expression of both CCR5 and CXCR4 in leukocytes (Steele et al., 2003). Taken together, these observations raise the possibility that repeated exposure to opioids and subsequent increases in chemokine receptor signaling might also be central to OIH.

We now demonstrate that many nociceptive neurons express functional receptors for a number of chemokines following systemic injection of morphine. Chemokine receptor signaling via the CXCR4 receptor may be central to OIH as the administration of the selective CXCR4 receptor antagonist, AMD3100, transiently reversed OIH in rats. Collectively, the data suggest that chemokine receptor antagonists represent a promising novel approach to the management of the side effects associated with long term opioids for chronic pain control.

Methods

Animals. Pathogen-free, adult female Sprague-Dawley rats (150–200 g; Harlan Laboratories, Madison, WI) were housed in temperature ($23 \pm 3^\circ\text{C}$) and light (12-h light: 12-h dark cycle; lights on at 07:00 h) controlled rooms with standard rodent chow and water available *ad libitum*. Experiments were performed during the light cycle. These experiments were approved by the Institutional Animal

Care and Use Committee of Loyola University, Chicago and Indiana University/Purdue University in Indianapolis. All procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health and the ethical guidelines of the International Association for the Study of Pain. All animals were randomly assigned to either treatment or control groups.

Drugs and method of administration The drugs, morphine sulfate salt and the bicyclam, AMD3100, were employed in this study. Morphine sulfate salt and AMD3100 were purchased from NIDA Drug Supply Program (Rockville, MD) and Sigma-Aldrich (St. Louis, MO), respectively. All drugs were freshly prepared in saline on the day of the experiment. Morphine sulfate- and vehicle-treated groups were given intraperitoneal (i.p.) injections once daily for 5 days of 10 mg/kg or saline (vehicle). After tactile hypernociception was established, animals were given an i.p. injection of AMD3100 (10 mg/kg) (**Fig. 3**). Previous nociceptive behavioral studies from our lab using AMD3100 at doses of (1, 5, 10, and 25 mg/kg) observed: no reversal of effect with 1 mg/kg, partial inconsistent effect with 5 mg/kg, reversal with 10 mg/kg, and reversal with side effects at 25 mg/kg (unpublished observations). Therefore, 10 mg/kg AMD3100 was selected for these studies.

Tactile Behavioral assessment Von Frey filaments were used to test mechanical sensitivity before, during and after cessation of morphine sulfate administration. Prior to initial von Frey tactile testing, all rodents were habituated to testing chambers for at least two days. Animals were tested for baseline

responses (BL) at least two times before undergoing the repeated morphine sulfate treatment (10 mg/kg, i.p. daily). Mechanical testing with von Frey filaments during the morphine sulfate dosing paradigm was limited to injection day (ID) 3. Behavioral assessment on ID3 occurred 18-20 hours after the ID2 morphine administration and before ID3 morphine or vehicle treatment (**Fig. 3**). Additional behavioral assessment following drug or vehicle administration occurred on post-injection day (PID) 1, 2, 3, 7, 14, 21, and 28. All behavioral testing was performed by laboratory assistants who were blinded to the experimental conditions and unfamiliar with the experimental aims.

The incidence of foot withdrawal in response to mechanical indentation of the plantar surface of each hindpaw was measured with a von Frey filament capable of exerting forces of 10, 20, 40, 60, 80 and 120 mN. These probes exhibit a uniform tip diameter (0.2 mm) and were applied to 6 designated loci distributed over the plantar surface of the foot (Ma et al., 2003). These 6 spots are representative of the distal nerve distributions of saphenous, tibial and sural nerves (medial to lateral) in the glabrous hindpaw. During each test, the rodent was placed in a transparent plastic cage with a floor of wire with $\sim 1 \times 1$ cm openings. The cage is elevated so that stimulation can be applied to each hind foot from beneath the rodent. The filaments were applied in order of ascending force. Each filament was applied alternately to each foot and to each locus. The duration of each stimulus was approximately 1 s and the inter-stimulus interval was approximately 10–15 s. The incidence of foot withdrawal is expressed as a percentage of the 6 applications of each stimulus and the percentage of

withdrawals was then plotted as a function of force (Bhangoo et al., 2007a; Ma et al., 2003). The von Frey withdrawal threshold was defined as the force that evoked a minimum detectable withdrawal observed on 50% of the tests given at the same force level. For cases in which none of the specific filaments used evoked withdrawals on exactly 50% of the tests, linear interpolation was used to define the threshold.

Foot withdrawal to thermal stimulus To evaluate the paw withdrawal threshold (PWT) to thermal stimulation, the Hargreaves' plantar test apparatus was used (Ugo Basile, Varese, Italy). Rats were placed on a 2-mm-thick glass floor; a mobile infrared heat generator with an aperture of 10 mm was aimed at the rat's hind paw under the floor. Following activation of the heat source (IR setting = 70), the reaction time (the withdrawal latency of the hindpaw) of the rat was recorded automatically. A shortening of the withdrawal latency indicated thermal hypernociception. The temperature of the glass floor was kept at 22.5–23.5 °C. Measurements of the withdrawal latency of the paw began after the rats were habituated to the testing environment. Animals were habituated to the testing apparatus each testing day for approximately 30 minutes. Five trials of thermal measurements were taken, at 5 min intervals, on each hind paw, and the initial pair of trial measurements was not used. The averages of the three remaining pairs of measurements taken were employed as data. Baseline recordings were taken 2-3 days prior to initiation of morphine dosing paradigm. Thermal behavior was measured on during dosing paradigm (ID4) and following the cessation of

the dosing paradigm (PID 3-9). The effect of the CXCR4 antagonist, AMD3100, on thermal behavior was determined PID7.

Assessment of Withdrawal Behavior Withdrawal behaviors were monitored by a trained observer for each rat 1 min every 10 min over a 30-min period.

Measurements recorded included locomotion: jumping, wet dog shakes, tremor, ptosis, and piloerection. The number of counts for each behavior over the 30 minute period was recorded and the average count among the treated animals was used as the data for each time point. Weight was also monitored during the testing period. Baseline recordings (BL) were taken for 2 days prior to initiation of morphine dosing paradigm. Withdrawal behavior was recorded following the cessation of morphine dosing paradigm (PID 1-8). The effect of the CXCR4 antagonist, AMD3100, on thermal behavior was determined PID7.

Tissue processing and immunocytochemistry for neural tissue. Morphine or control treatments rats' lumbar (L₃-L₆) DRG tissue was collected after animals were sacrificed and transcardially-perfused with saline followed by fixative. Fixed tissue was then embedded for sectioning and processed using immunocytochemical methodologies commonly used in this lab (Bhangoo et al., 2007a). Tissue sections from L₄ and L₅ were used in immunocytochemical experiments. Tissue sections were blocked with natural horse serum blocking buffer (SuperBlock® Blocking Buffer in PBS (Thermo Scientific, Rockford, IL, Natural Horse Serum (3% v/v), Triton X (0.4% v/v)). Primary antisera used was the anti-CXCR4 rat monoclonal antibody, 2B11 (1:20,000 dilution; BD Biosciences, San Jose, CA) which binds to both human and mouse CXCR4

(Forster et al., 1998; Schabath et al., 1999). CXCR4 anti-body was dissolved in blocking buffer and incubated overnight. After primary incubation, slides were incubated in secondary antibodies (1:1000 dilution; anti-rat made in donkey conjugated to CY3, Jackson ImmunoResearch, West Grove, PA).

Images were collected with a DeltaVision microscope (Applied Precision) equipped with a digital camera (CoolSNAP HQ; Photometrics), using a 1.4-numerical aperture (NA) 20x objective lens, and were deconvolved with SoftWoRx deconvolution software (Applied Precision).

Preparation of acutely dissociated dorsal root ganglion neurons. The L1-L6 DRGs were acutely dissociated using methods described by Ma and LaMotte (Ma and LaMotte, 2005). Briefly, L1-L6 DRGs were removed from naive or morphine-treated animals four to six days following the last morphine injection. The DRGs were treated with collagenase A and collagenase D in HBSS for 20 minutes (1 mg/ml; Roche Applied Science, Indianapolis, IN), followed by treatment with papain (30 units/ml, Worthington Biochemical, Lakewood, NJ) in HBSS containing .5 mM EDTA and cysteine at 35°C. The cells were then dissociated via mechanical trituration in culture media containing 1 mg/ml bovine serum albumin and trypsin inhibitor (1 mg/ml, Sigma, St. Louis MO). The culture media was Ham's F12 mixture, supplemented with 10% fetal bovine serum, penicillin and streptomycin (100 ug/ml and 100 U/ml) and N2 (Life Technologies). The cells were then plated on coverslips coated with poly-L lysine and laminin (1 mg/ml) and incubated for 2-3 hours before more culture media was added to the

wells. The cells were then allowed to sit undisturbed for 12–15 hours to adhere at 37°C (with 5% CO₂).

Intracellular Ca²⁺ imaging. The dissociated DRG cells were loaded with fura-2 AM (3 μ M, Molecular Probes/Invitrogen Corporation, Carlsbad CA) for 25 minutes at room temperature in a balanced sterile salt solution (BSS) [NaCl (140 mM), Hepes (10 mM), CaCl₂ (2 mM), MgCl₂ (1 mM), Glucose (10 mM), KCl (5 mM)]. The cells were rinsed with the BSS and mounted onto a chamber that was placed onto the inverted microscope. Intracellular calcium was measured by digital video microfluorometry with an intensified CCD camera coupled to a microscope and MetaFluor software (Molecular Devices Corporation, Downingtown, PA). Cells were illuminated with a 150 W xenon arc lamp, and the excitation wavelengths of the fura-2 (340/380 nm) were selected by a filter changer. Sterile solution was applied to cells prior to chemokine application, any cells that responded to buffer alone were not used in chemokine responsive counts. Chemokines were applied directly into the coverslip bathing solution. If no response was seen within 1 minute, the chemokine was washed out. For all experiments, MCP1, SDF1, regulated upon activation, normal T cell expressed and secreted (RANTES/CCL5), and interferon-gamma-induced protein (IP10/CXCL10) were added to the cells in random order, after which capsaicin (3nM), high K⁺ (50 μ M) and ATP (3nM) were added. The chemokines used were purchased from R & D Systems (Minneapolis, MN; <1.0 endotoxin per 1 μ g of the protein by the LAL method), and all were used at a concentration of 100 nM to ensure maximal activation (Bhangoo et al 2007a; Bhangoo et al 2007b).

Chemokines were reconstituted in sterile 0.1%BSA/PBS, and aliquots were stored at -20°C. Calcium imaging traces were analyzed by two independent analyzers and only responses that were in agreement between two individuals were used in the counts.

***In situ* hybridization.** *In situ* hybridization histochemistry for chemokine receptors was performed using digoxigenin-labeled riboprobes. Treated and non-treated rodents were sacrificed using carbon monoxide. Lumbar DRGs from the injected and control animals were rapidly removed, embedded in OCT compound (Tissue Tek, Ted Pella, Inc., Redding, CA) and frozen. L₄ and L₅ DRG sections were cut serially at 12 μ m. The SDF1 probes were generated as described previously (Lu et al., 2002). Signals were visualized by using NBT/BCIP reagents (Roche Applied Science, Indianapolis, IN) in the dark for 2–20 h depending upon the abundance of the RNA. The *in situ* image was captured using a Retiga EX charge-coupled device camera (Q-imaging, Burnaby, BC).

Plasmid construction. To make chemokine-fluorescent protein fusion constructs, MCP1 and SDF1-alpha protein coding sequence was cloned into pEGFP-N1 or pmCherry-N1 (Clontech).

F11 Culture Conditions. F11 cells (a mouse N18TG2 neuroblastoma X rat DRG sensory neuron hybrid cell line) were grown as monolayers either in 100-mm plastic dishes under 5% CO₂ in Ham's F-12 medium supplemented with 20% fetal bovine serum (Hyclone), 100 pM hypoxanthine/ 1 pM aminopterin/ 12 pA4 thymidine, and 50 IU/ml of penicillin/streptomycin. Cells were fed every other day for several days preceding an experiment with Ham's F-12 medium

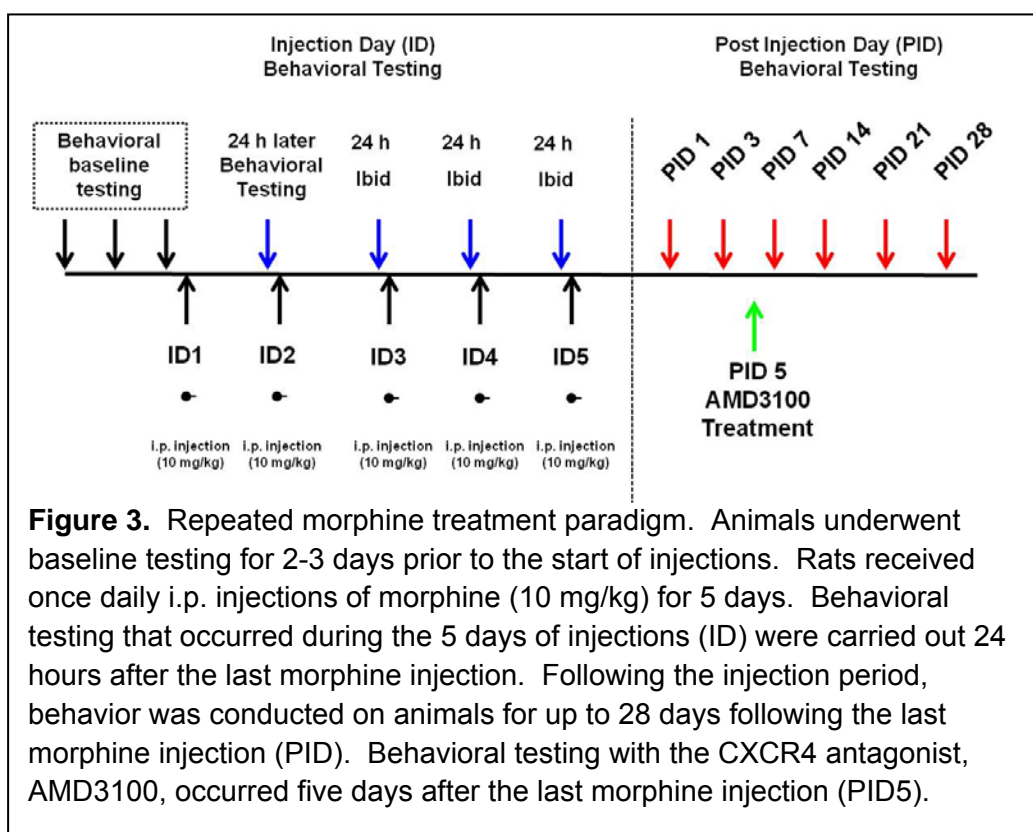
supplemented with 1% fetal bovine serum, 50 ng/ml of NGF, 2 pM retinoic acid, 0.5 mM dibutyryl cyclic AMP, 10 pM 3-isobutyl-1-methylxanthine (IBMX), a 1:500 dilution of 2.5 mg/ml of bovine insulin, a 1:100 dilution of 10 mg/ml of transferrin, and 50 IU/ml of penicillin/streptomycin.

Enzyme-linked immunosorbent assay (ELISA). Constitutive and regulated release of MCP1-RFP (mCherry) and SDF1-RFP (mCherry) was measured by sandwich ELISA. F11 DRG neuronal cells were transfected with MCP1-RFP or SDF1-RFP. 24 h after the transfection, cells were placed under differentiating conditions and allowed to differentiate for 48 h. When cells were fully differentiated, culture medium was replaced with balanced salt solution (BSS) containing either 5 mM (normal) or 50 mM KCl (depolarizing). Normal BSS (145 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂) and depolarizing BSS (100 mM NaCl, 50 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂) had the same osmolarity. After 30 min, released MCP1-RFP or SDF1-RFP was measured from supernatant by sandwich ELISA. A polyclonal anti-RFP antibody (Abcam ab34771) was used as the capture antibody (1:50,000). Chemokine-specific antibodies were used as the detecting antibodies: for SDF1, a mouse monoclonal anti-SDF1 antibody (Santa Cruz sc-74271); for MCP1, a goat polyclonal antibody (Santa Cruz sc-1785).

Statistics. Data for sandwich ELISA were presented as mean \pm SEM and analyzed by one way ANOVA followed by Newman-Keuls multiple comparison tests. Prism 5 (GraphPad, LaJolla, CA) was used to determine the statistical significance of differences in the mean threshold forces for foot withdrawal to

punctate indentation as a function of time and between experimental groups by means of repeated measures analyses of variance (RMANOVA) followed by post hoc pairwise comparisons (Tukey method). Statistical significance was set at $p < 0.05$. GraphPad Software (LaJolla, CA) was used to determine the statistical significance of differences in calcium response among naïve and treatment groups using Chi-square test with Yates correction with $p < 0.05$ set as statistical significance.

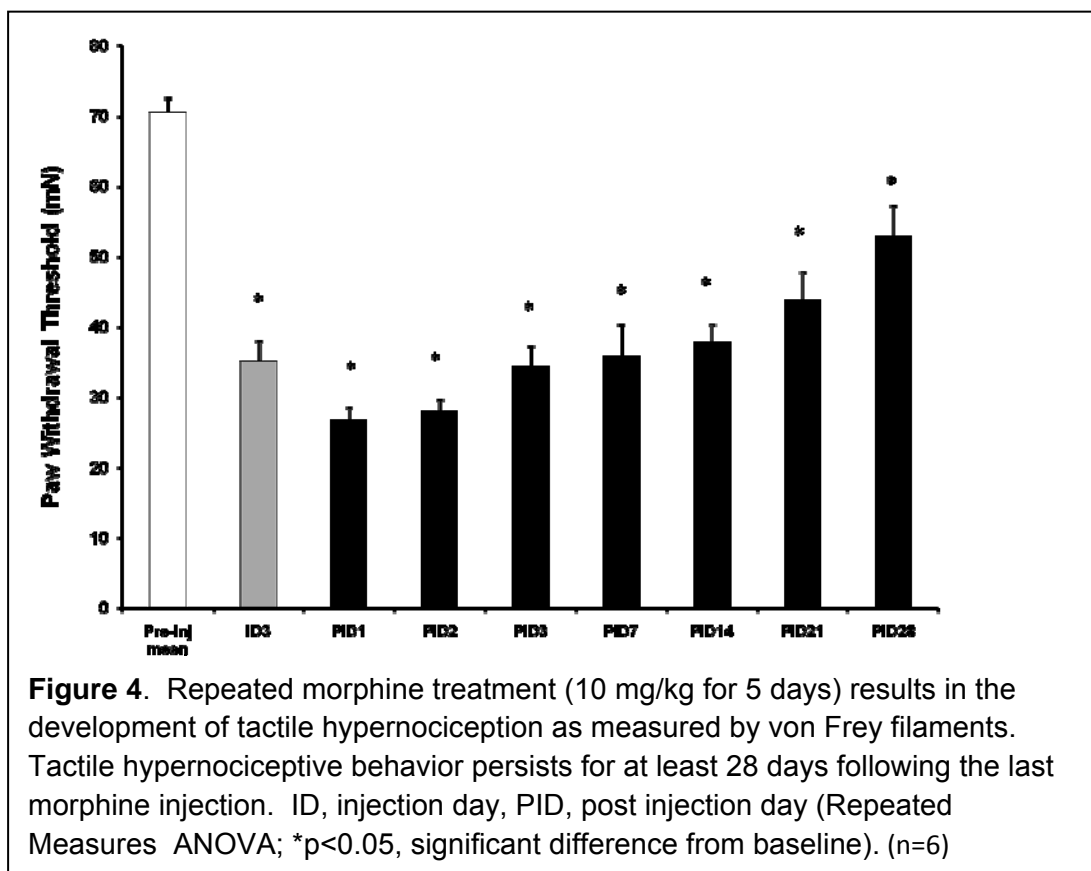
Results



Repeated morphine treatment leads to tactile hypernociception

Initial experiments were conducted in order to determine the ability of our morphine dosing paradigm to induce nociceptive behavior as measured by tactile

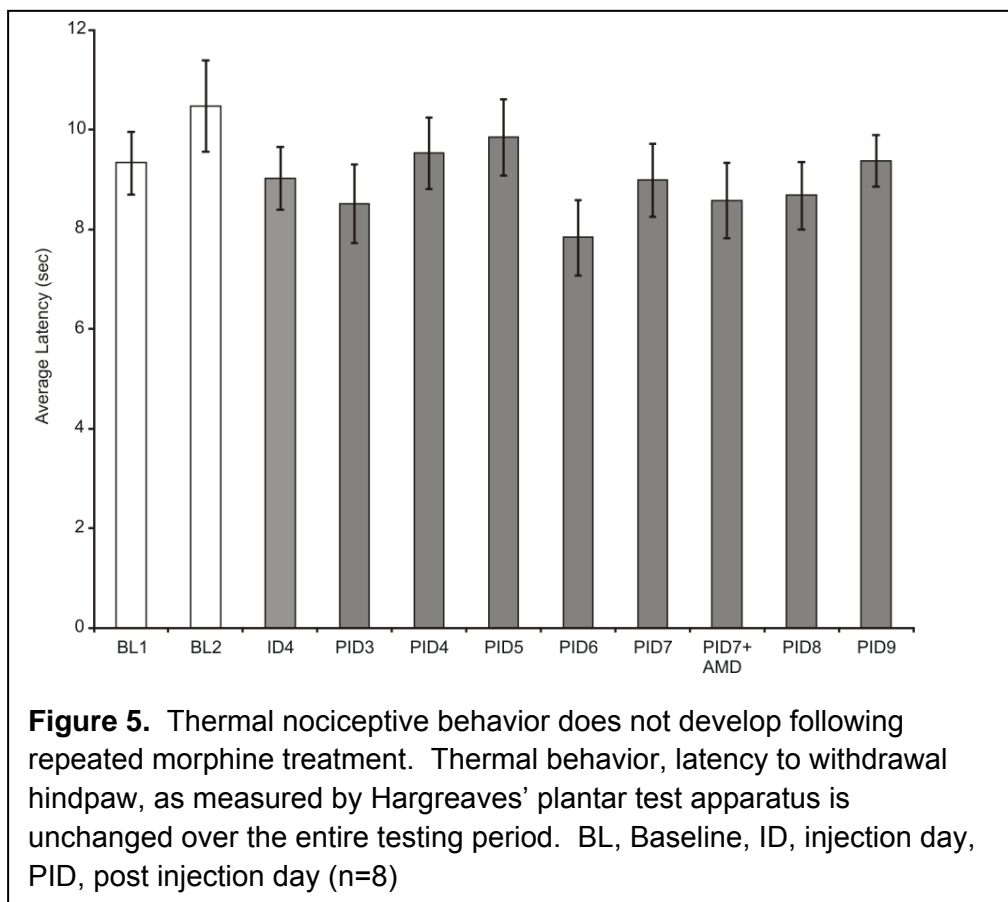
and thermal assessment. Tactile hypernociception as measured by von Frey filaments is a characteristic behavioral response that develops in rodents following repeated administration of morphine (Celerier et al., 2000; Gardell et al., 2002). Our behavioral assessment of tactile hypernociception was performed prior to the start of the injection paradigm, during the 5 day dosing regimen, and for 28 days following the repeated morphine treatment paradigm (**Fig.4**). In our experiments, the mean paw withdrawal threshold (PWT) of the tested hind paws exhibited a decrease after only two daily morphine injections relative to pre-injection baseline PWT (ID3; 70.5 ± 2.1 mN to 35.1 ± 2.9 mN; $n=12$; $p<0.0001$). Statistically significant decreases in PWT were maintained until at least PID28 (**Fig. 2**).



The changes in PWT observed at ID3 suggest that this nociceptive behavior is unaffiliated with morphine withdrawal signs such as jumping or wet dog shakes. Opioid withdrawal behaviors were observed between 24-48 hours following the last morphine injection and continued to be measured for 9 days following the cessation of the morphine dosing paradigm. Alterations in the paw withdrawal latency evoked by thermal stimulation were not observed with this dosing paradigm (**Fig. 5**).

These experiments demonstrated our repeated morphine dosing paradigm is able to induce tactile nociceptive behavior that persists for at least 28 days

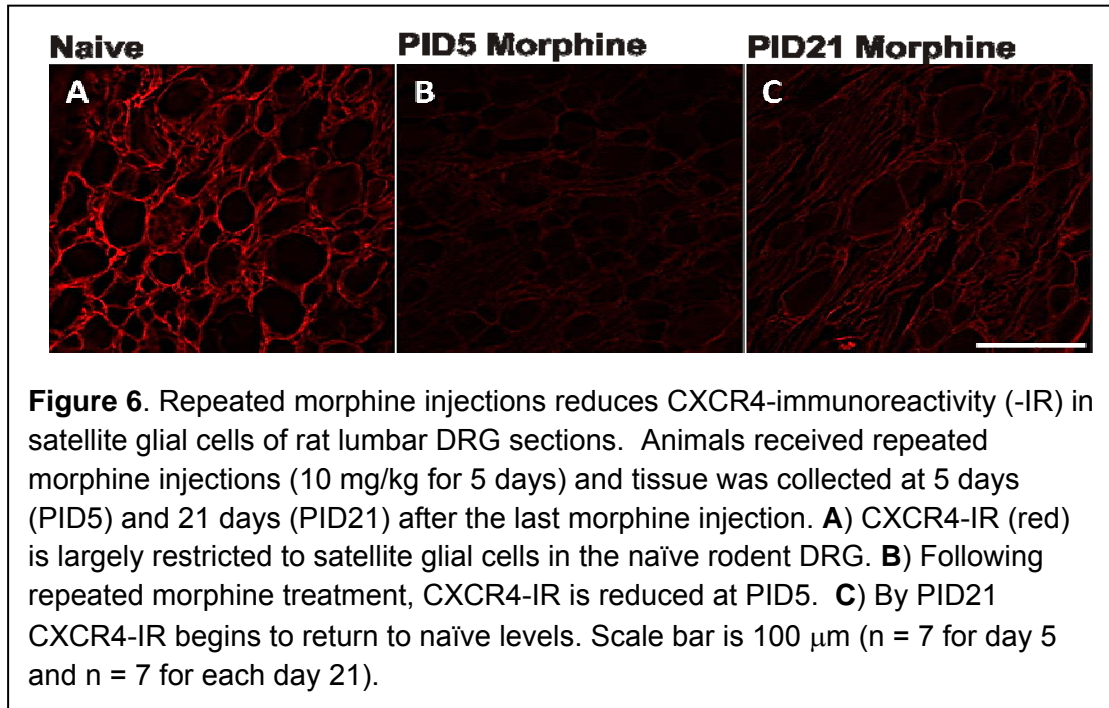
following the last morphine injection. However, thermal nociceptive behavior was not observed.



Repeated morphine treatment decreases CXCR4 immunoreactivity in the rat DRG.

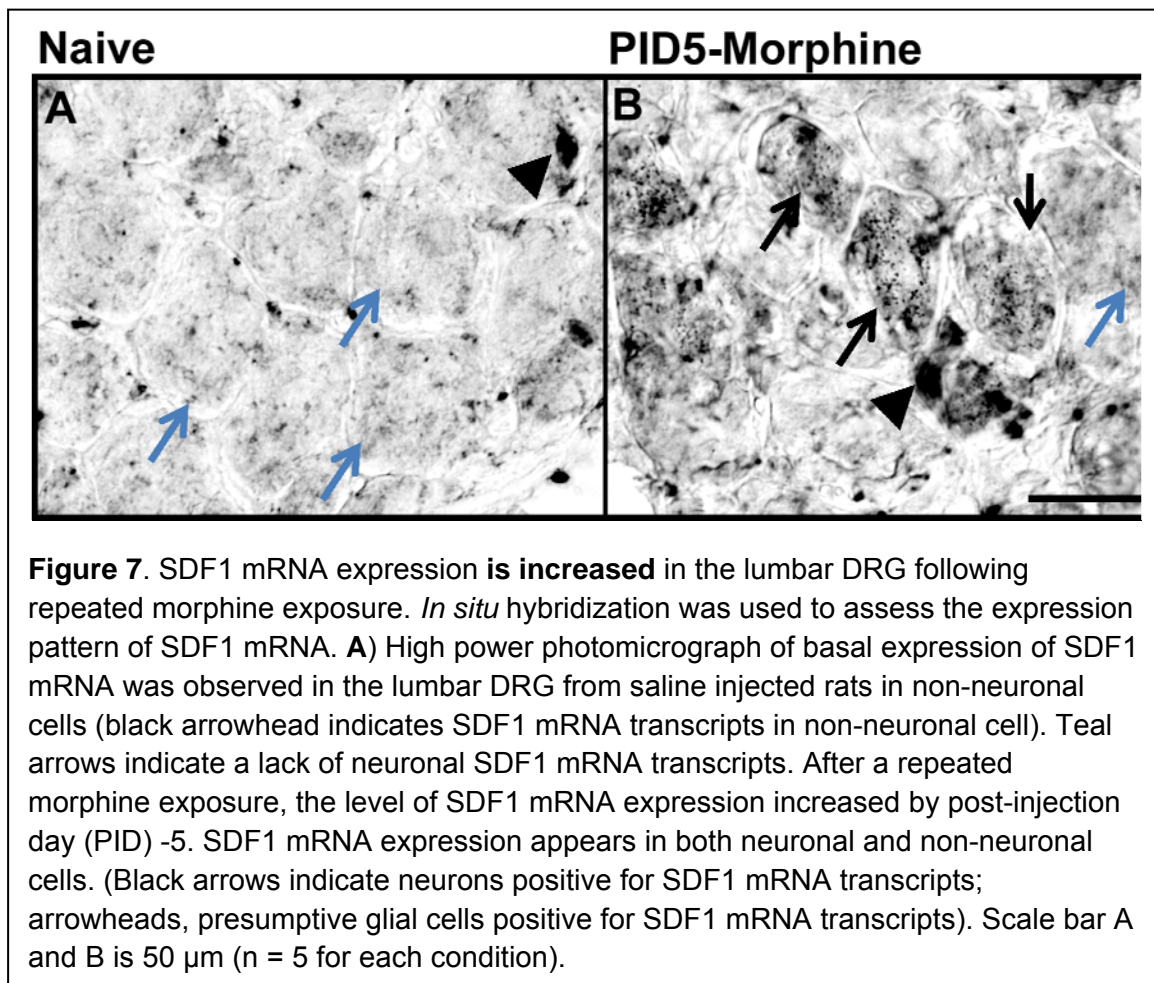
To assess the changes in CXCR4 expression in the rodent DRG following repeated morphine administration, immunocytochemistry experiments were conducted on DRG tissue sections using an antibody against the CXCR4 receptor.

The CXCR4 antibody binds the N-glycosylation site g1 of human CXCR4. Although this site does not influence HIV-1 coreceptor function (Huskens et al., 2007), this antibody is an effective neutralizing antibody in tumor formation and angiogenesis (Kato and Kato, 2010). In the adult rat dorsal root ganglia (DRG), CXCR4 expression is largely limited to presumptive nonmyelinating satellite glial cells (SGCs) of the DRG based on anatomical locale (**Fig. 6A**). In addition to the SGCs, an occasional neuron was also observed to express the CXCR4. This expression pattern in the DRG coincides with CXCR4 mRNA expression pattern seen previously (Bhangoo et al 2007). By comparison, very few CXCR4-immunoreactive, nonmyelinating SGCs were evident following repeated morphine treatment at PID5 (**Fig. 6B**). By PID21, CXCR4 immunoreactivity (-ir) in the nonmyelinating SGCs of the morphine treated rats was again evident (**Fig. 6C**). Concurrent with return of CXCR4-ir, SGCs in the DRG at PID21 was the gradual return of PWT to pre-treatment BL thresholds (**Fig. 4**).



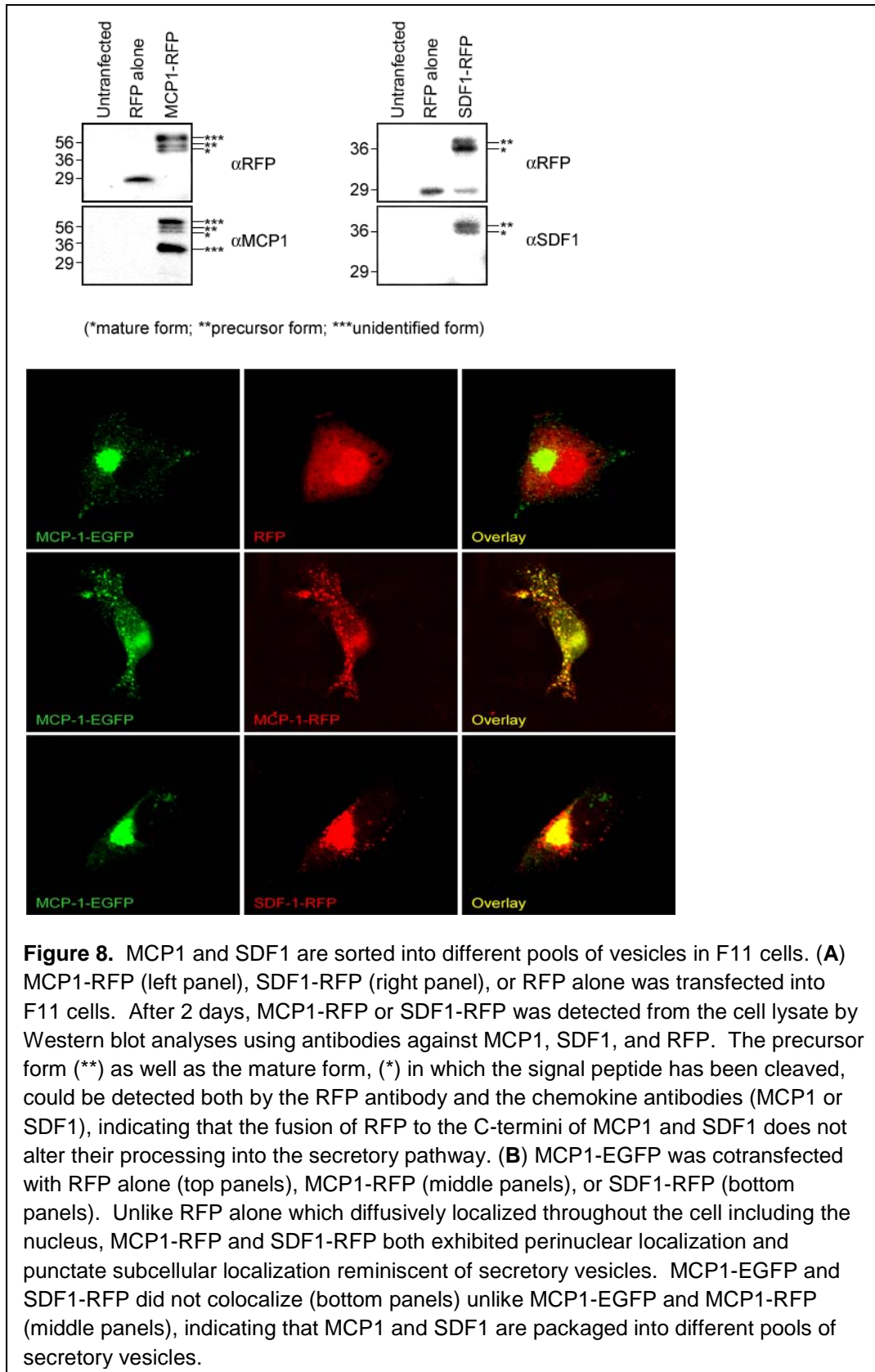
SDF1 mRNA is increased in sensory neurons following repeated morphine treatment and the protein is tonically released.

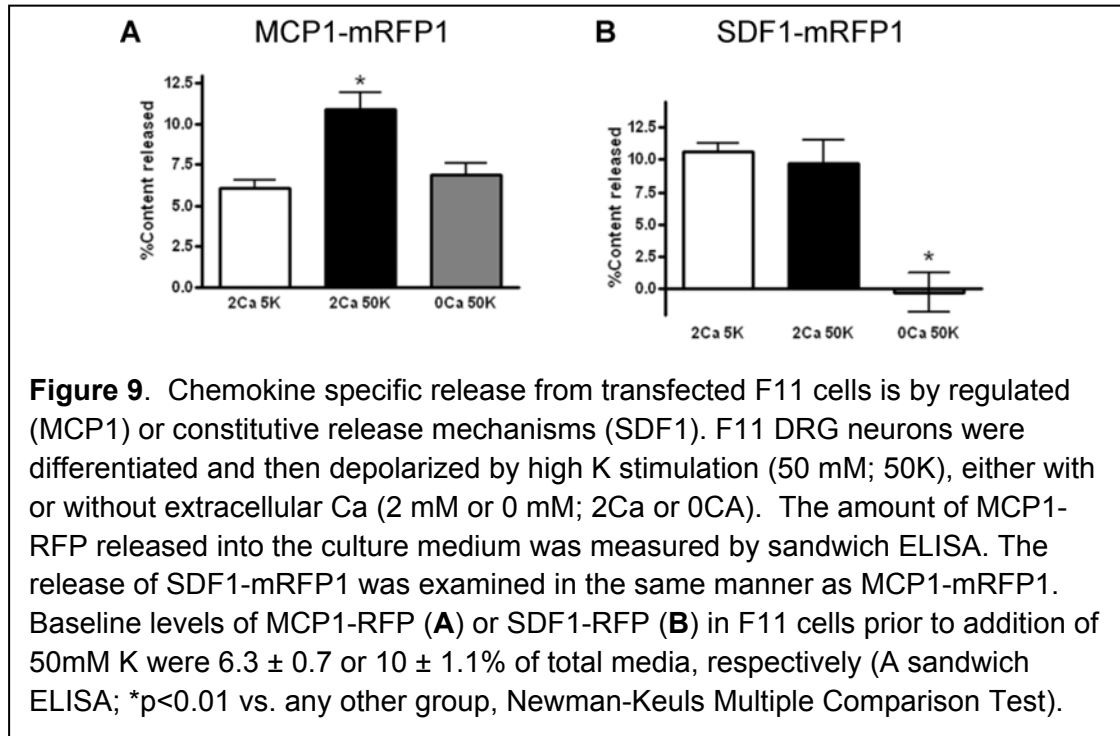
Given the apparent decline of CXCR4-ir in the DRG following the repeated morphine treatment paradigm, we determined whether SDF1 mRNA expression in the lumbar DRG was also altered by the dosing paradigm. We observed cellular expression patterns of SDF1 mRNA transcripts by *in situ* hybridization using digoxigenin-labeled riboprobes in relatively few cells of the saline-treated DRG (**Fig. 7A**). Following repeated morphine treatment both non-neuronal cells and numerous sensory neurons exhibited SDF1 mRNA transcripts (**Fig.7B**).



The observation that SDF1 is expressed in DRG neurons following repeated morphine treatment raises the possibility that the ligand may be tonically released from these cells. Hosung Jung from Dr. Richard Miller's lab therefore examined the release of SDF1 using the F11 cell line. This cell line was derived from DRG neurons and maintains many of the differentiated properties of these cells (Platika et al., 1985). We compared the characteristics of SDF1 release to those of MCP1/CCL2, another chemokine that has been shown to be expressed and released by DRG neurons (Jung et al., 2008). Following the expression of chemokine fluorescent fusion proteins (SDF1-RFP and MCP1-

EGFP) we noted that the two chemokines localized to different sets of secretory vesicles (**Fig. 8**). We measured constitutive and K⁺ depolarization induced release of each chemokine from F11 cells using a sandwich ELISA, and observed that the patterns of release for the two chemokines were also different. Release of MCP1-RFP from differentiated F11 cells was significantly increased by depolarizing medium containing high K⁺ (**Fig 9A**). However, the release of SDF1-RFP was quite apparent under non depolarizing conditions and was not increased further by K⁺ depolarization (**Fig 9B**), suggesting that most SDF1 release was constitutive whereas a significant portion of MCP1 release was regulated by neuronal depolarization. This result implies that once the expression of SDF1 has been increased in DRG neurons (**see Fig 7**); it will be constitutively released from these cells.





Repeated morphine treatment increases functional chemokine receptor expression by capsaicin sensitive DRG neurons.

To further investigate the status of functional CXCR4 receptor expression in the DRG following repeated morphine treatment, we utilized Ca^{2+} imaging studies in acutely dissociated DRGs derived from animals subjected to repeated morphine conditions and naive controls. The time points used corresponded to time points used for immunohistochemical and behavioral assessment (**Fig 6**). The acutely dissociated DRG preparations were categorized into three neuronal and non-neuronal cell types: non-capsaicin sensitive neurons (high K and ATP responsive), capsaicin sensitive neurons (capsaicin, high K, and ATP responsive), and glia (ATP responsive only). These cell response criteria were chosen strictly as an indicator of the types of cells that may be affected by the

repeated morphine treatment paradigm. The tested chemokines were selected so as to activate a wide spectrum of chemokine receptors known to be expressed by neurons and non-neuronal cells (CXCR4-SDF1/CXCL12, CXCR3-IP10/CXCL10, CCR2-MCP1/CCL2, CCR5-RANTES/CCL5). The chemokine concentration used for these experiments were based on their maximally effective concentrations using our previous observations on acutely dissociated DRGs (Bhangoo et al., 2007a; Bhangoo et al., 2007b).

	<u>Naïve</u>			<u>Morphine-Treated</u>		
	<i>Non-capsaicin sensitive neurons</i>	<i>Capsaicin -sensitive neurons</i>	<i>Glia</i>	<i>Non-capsaicin sensitive neurons</i>	<i>Capsaicin -sensitive neurons</i>	<i>Glia</i>
SDF1	7% (6/85)	7% (5/72)	21% (9/44)	15% (17/112)	34% (25/73)**	27% (13/49)
IP-10	4% (3/85)	0% (0/72)	2% (1/44)	13% (14/112)*	25% (18/73)**	8% (4/49)
MCP1	4% (3/85)	6% (4/72)	9% (4/44)	16% (18/112)**	29% (21/73)**	25% (12/49)
RANTES	14% (12/85)	4% (6/72)	21% (9/44)	17% (19/112)	29% (21/73)**	12% (6/49)

Table 2. Repeated morphine treatment increases nociceptive neurons chemokine calcium responsiveness. Daily morphine injections (10 mg/kg for 5 days) were administered to animals. Lumbar DRGs were acutely dissociated from these animals 4-6 days following the last morphine injection. The most significant increase in chemokine calcium responsiveness occurred in the nociceptive neurons (** p<0.01, * p<0.05, Chi-square with Yates correction). (naïve, n=8, morphine-treated n=6)

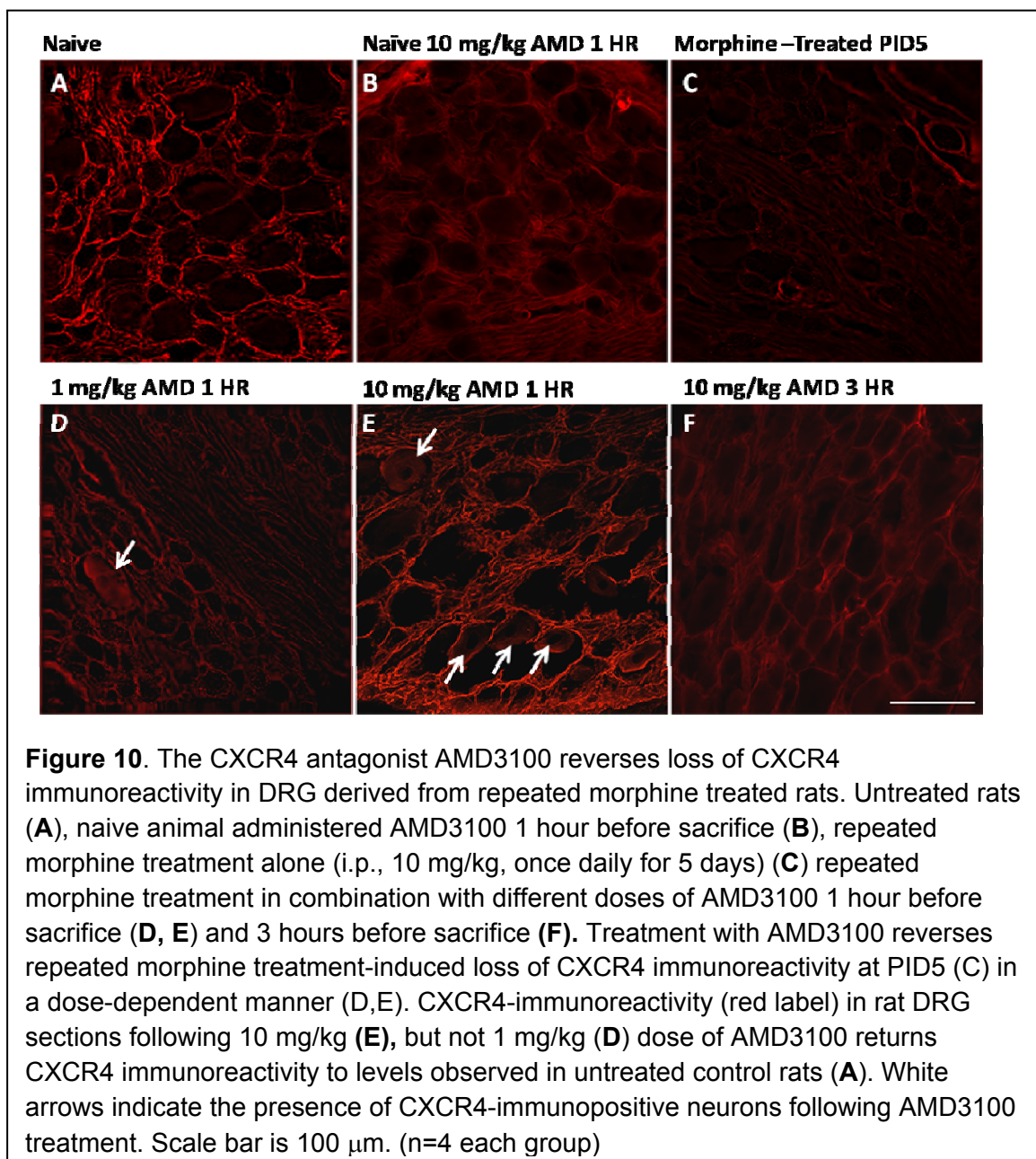
Application of all tested chemokines produced $[Ca^{2+}]_i$ changes in small numbers of neuronal and non-neuronal populations of cells derived from control DRGs (**Table 2**). Following exposure to repeated morphine treatment, we observed a significant increase in the chemokine responsiveness of non-

capsaicin sensitive and capsaicin sensitive neurons. This included a robust increase in SDF1 responsive capsaicin sensitive neurons ($p < 0.0001$). Hence it appears that DRG nociceptive neurons express more functional chemokine receptors, including CXCR4 receptors, following the repeated morphine treatment paradigm (**Table 2**).

Reduced CXCR4 expression following repeated morphine treatment is abolished by AMD3100 treatment

Given that tonic activation of CXCR4 by SDF1 leads to internalization of both chemokine and receptor (Burger and Kipps, 2006), it is entirely possible that the constitutive release of neuronal SDF1 and subsequent neuronal signaling via CXCR4 may result in diminished evidence of CXCR4-ir in the sensory ganglia of morphine treated rats (**Fig 100C**). This event would not be unlike previous reports in the dentate gyrus (Bhattacharyya et al., 2008; Kolodziej et al., 2008). Additional studies have shown that the binding capabilities of the CXCR4 antibody utilized for these studies can compete with SDF1 binding sites (Dubeykovskaya et al., 2009). To test this possibility we intraperitoneally administered the CXCR4 antagonist, AMD3100, at doses of 1 and 10 mg/kg and sacrificed the animals one hour later. Overall increases in CXCR4-ir binding were observed following AMD3100 administration in morphine treated animals at 10 mg/kg (**Fig. 10E**), but not 1 mg/kg (**Fig. 10D**). As evidence of the *de novo* SDF1 signaling via CXCR4, CXCR4-ir was also observed in numerous neurons (**Fig. 10E**). This data provides further support for the increased functional CXCR4 receptors observed in neurons (see **Table 2**). Three hours after

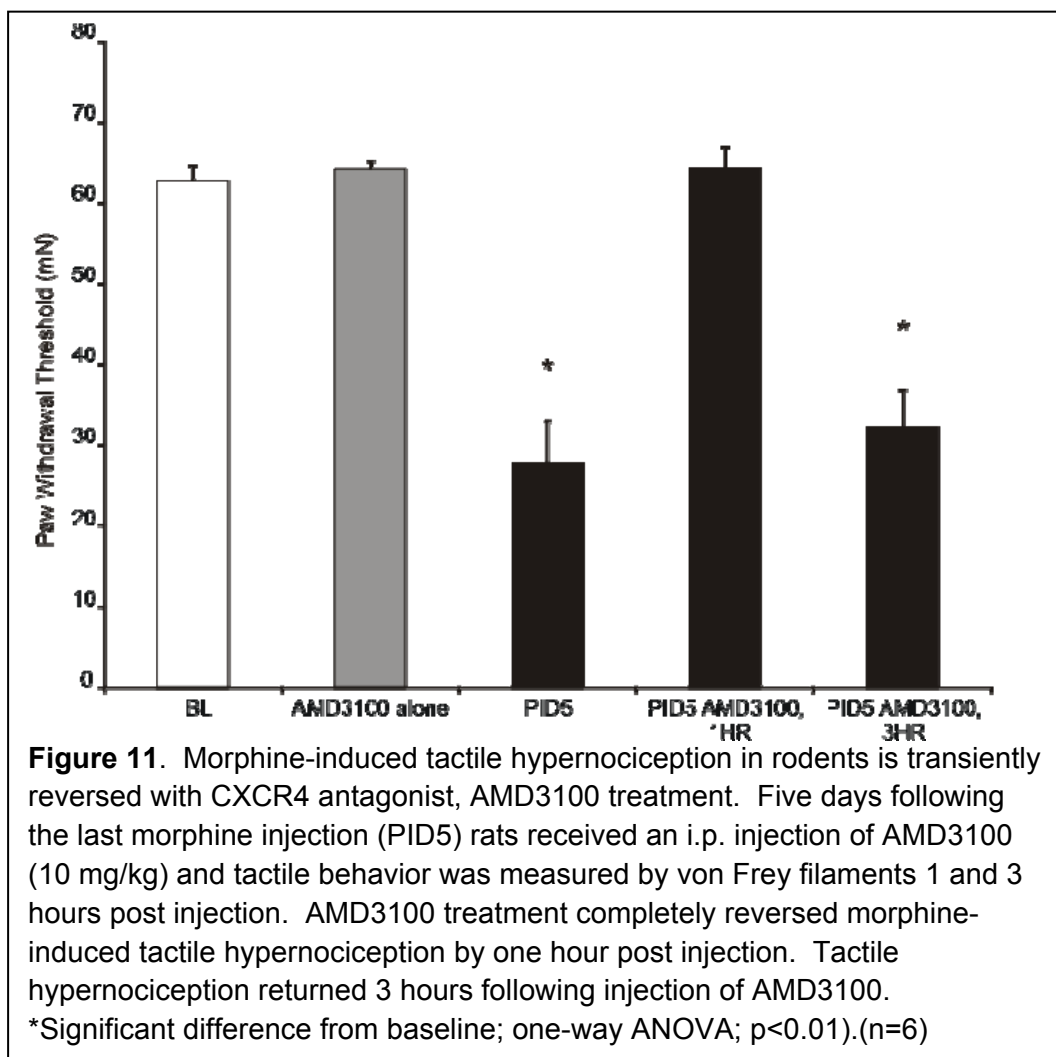
AMD3100 administration, the CXCR4-ir was again qualitatively decreased in the DRG (**Fig. 10F**). Following administration of AMD3100 in the naïve animal (**Fig. 10B**), there was a noticeable decrease in CXCR4-ir when compared with the naïve animal (**Fig. 10A**). This is likely attributed to the competition that exists between AMD3100 and CXCR4 antibody for available receptor binding sites.



Decreased tactile hypernociception following intraperitoneal injection of CXCR4 antagonist, AMD3100.

To determine whether SDF1/CXCR4 signaling was involved in OIH, we administered a single systemic dose of either vehicle or 10 mg/kg AMD3100 i.p.

at PID5. Systemic injection of AMD3100 (10 mg/kg) in naïve rodents did not alter baseline PWT (70.5 ± 2.1 mN; **Fig 11**). Vehicle injections in morphine treated rats did not alter PWT (data not shown). However, 1 hour after AMD3100 was administered to morphine treated rats, PWT returned to baseline (69.25 ± 2.75 mN; $p < 0.01$). The rapid onset of AMD3100 was short lived as PWTs returned to pre-dosing levels by three hours (35.25 ± 4.59 mN) (**Fig 11**). The return of morphine-induced behavior 3 hours post AMD3100 injection coincides with the returned loss of CXCR4-ir observed 3 hours post AMD3100 injection in rat DRG sections (**Fig 10F**).



Discussion

The experiments reported here demonstrate that following repeated morphine exposure, rodents exhibited a prolonged tactile hypernociception. This change in paw withdrawal threshold was maintained at least until PID28. Importantly, morphine-induced tactile hypernociception could be transiently reversed by a chemokine receptor antagonist that is selective for CXCR4 receptors. These results provide the first demonstration that morphine induced

tactile hypernociception behavior in the rodent appears to be dependent on activation of CXCR4 receptors. Thus, morphine induced SDF1 signaling via CXCR4 receptors appears to change the balance between opioid analgesia and hypernociception.

OIH has been observed both clinically (Angst et al., 2003; Arner et al., 1988; Singla et al., 2007) and experimentally (Laulin et al., 1999; Woolf, 1981). Many explanations for this phenomenon have been suggested which have centered largely on changes within the central nervous system. These potential mechanisms include enhanced production/release of glutamate and neuropeptides in the spinal cord (Belanger et al., 2002; Ibuki et al., 2003; Mao et al., 2002), protein kinase C γ -induced signaling (Lim et al., 2005), spinal Ca^{2+} /calmodulin-dependent protein kinase II α activity (Chen et al., 2010), enhanced descending facilitation of nociceptive pathways from the rostral ventromedial medulla (Vanderah et al., 2001) and activation of non-classical opioid receptors (Lewis et al., 2010). Alternative peripheral mechanisms also include sensitization of peripheral nociceptors (Aley and Levine, 1997; Liang et al., 2008).

The mechanisms responsible for morphine induced SDF1/CXCR4 signaling in primary sensory neurons of the DRG are largely unknown. Thus, the effects we have observed might be downstream of morphine's interactions with μ -opioid receptors or possibly through interactions with TLR4 as has been recently suggested (Hutchinson et al., 2010). A number studies have been

conducted demonstrating the ability of chemokines and opioid agonists administration to induce heterologous desensitization to their respective systems (Szabo et al., 2002; Chen et al., 2007b). These studies have clearly shown that there is a relationship between the chemokine and opioid signaling pathways during acute administration. However, our results expand on these studies by suggesting that following the chronic administration of morphine produces an alteration in sensory neuron SDF1/CXCR4 signaling that lasts for at least 5 days after the last opioid injection.

With respect to morphine induced changes in CXCR4 activation, there appear to be two important changes in the status of SDF1/CXCR4 signaling within the DRG. First, it appears that greater CXCR4 signaling occurs following the dosing regimen. This is indicated by the fact that during OIH there is an AMD3100 reversible decline in CXCR4-ir in the DRG, presumably resulting from SDF1 activation of CXCR4 receptors followed by their internalization and recycling mechanisms, as previously observed in the dentate gyrus (Bhattacharyya et al., 2008; Kolodziej et al., 2008). Secondly, the increased SDF1/CXCR4 activation could result from enhanced tonic release of neuronal SDF1 whose expression was upregulated under these conditions, or some postsynaptic effect of morphine which produces enhanced CXCR4 desensitization in response to tonically released SDF1. In particular, the Ca^{2+} imaging experiments and the immunohistochemistry studies following AMD3100 administration clearly demonstrate that morphine treatment results in considerable degree of upregulated expression of CXCR4 by DRG nociceptors.

Our results highlight the rapid timecourse of CXCR4 downregulation and recycling that occurs in the DRG and other cell types. Numerous studies have demonstrated that the time course and extent of CXCR4 recycling in different cell types is subject to a very large number of factors that can interact with the receptor and regulate the different stages of endocytosis, and recycling or degradation (Tarasova et al., 1998; Zhang et al., 2004b). Thus it is becoming clear that regulating the levels of CXCR4 cell surface expression is one important mechanism of adjusting the signaling possibilities through this pathway. As activation of chemokine receptors expressed by DRG neurons produces excitation (White et al., 2007), it is likely the activation of these receptors by SDF1 contributes to the ectopic excitability of these neurons and produces AMD3100 reversible tactile hypernociception. The transient effect of AMD3100 may be explained by the short half life of 0.9 hours following a single administration (Hendrix et al., 2000). To this end, our observations support the growing body of literature that chemokines can act as neurotransmitters under some circumstances (White et al., 2007).

In the case of SDF1, its release mechanism may be unusual as it does not seem to require a depolarization induced increase in Ca^{2+} , in contrast to the depolarization dependent release of MCP1. The different release mechanisms may be due to the observation that the two chemokines appear to be stored in separate subcellular compartments. Thus, it is possible that the SDF1 storage vesicles may be released by lower levels of Ca^{2+} or by low Ca^{2+} in cooperation with some other signaling mechanism. We demonstrated that morphine will

increase the expression of SDF1 within DRG neurons. According to our data increased concentrations of SDF1 within DRG neurons should result in increased tonic release of the chemokine. The fact that appreciable levels of SDF1 may be tonically released both in the DRG (data herein) and the dentate gyrus suggest that SDF1 may generally be secreted in this way when utilized in the nervous system (Bhattacharyya et al., 2008; Kolodziej et al., 2008). Until recently, CXCR4 was known to be the only receptor for SDF1. This idea was challenged when the chemokine receptor, CXCR7, was shown to bind SDF1 (Balabanian et al., 2005). Initially described as a scavenger receptor, more recent interactions describe CXCR7 as possibly moderating the response of CXCR4 to SDF1 by internalizing the ligand (Zabel et al., 2009). Whether SDF1/CXCR7 activation serves to modulate OIH is unknown. However, we have observed that CXCR7 is expressed in the DRG of adult mice (unpublished observations) and so this remains a possibility.

In conclusion, ongoing SDF1/CXCR4 signaling within sensory neurons provides a mechanistic basis for understanding OIH modifications within the nervous system. Beyond its signaling relevance in the sensory neuron, the relationship between neuronal expression of SDF1/CXCR4 and tactile hypernociception in the rodent may imply that chemokine-sensitized sensory neurons may serve as an excitatory signal central to OIH. Thus, OIH represents another example of chronic pain behavior where chemokine signaling in DRG neurons has been observed to be upregulated (White et al., 2009), further

highlighting the potential role of chemokine signaling in the generation of chronic pain.

CHAPTER THREE

THE ROLE OF OPIOID AND NON-OPIOID RECEPTOR SIGNALING IN SDF1/CXCR4 INDUCED CHANGES IN THE DORSAL ROOT GANGLION

Overview

Opioid analgesics such as morphine represent one of the most effective treatment options for moderate to severe pain. Clinical usage of opioid analgesics while effective can also be accompanied by a number of side effects such as respiratory depression, urinary retention, or dependence. Individuals taking opioids such as, morphine can also develop increased pain sensitivity referred to as opioid induced hypernociception (OIH). The mechanism behind the development of OIH remains largely unknown. However, recent work in our lab has implicated the upregulation of SDF1/CXCR4 signaling in OIH. The purpose of the current study was to identify the receptor activation responsible for the upregulation of chemokines in the DRG of animals exhibiting OIH. In order to dissect the receptor activation events responsible we used pharmacological tools with distinct receptor affinity patterns. In order to test the role of non-opioid signaling we used the morphine metabolite, morphine 3- β -D-glucuronide (M3G), and morphine co-administered with the non-selective opioid antagonist naltrexone. The selective mu opioid receptor (MOR) agonist, D-Ala², N-MePhe⁴, Gly-ol-enkephalin (DAMGO), was employed to demonstrate the role

of MOR in changes in SDF1/CXCR4. We demonstrate that OIH is induced through opioid and non-opioid signaling events as repeated administration of DAMGO, M3G, and morphine co-administered with naloxone all induced nociceptive behavior. However, only repeated DAMGO administration is able to induce changes in SDF1/CXCR4 signaling in the rodent DRG. Changes in SDF1/CXCR4 signaling within the DRG are indicated by a decrease in CXCR4-immunoreactivity that is reversed by the CXCR4 antagonist, AMD3100. Additionally, DAMGO-induced nociceptive behavior is partially reversed with the administration of AMD3100. The data presented implicate MOR activation as responsible for changes in SDF1/CXCR4 signaling previously witnessed with morphine. Furthermore, the data demonstrate that the mechanism responsible for inducing OIH involves both opioid and non-opioid receptor signaling events. Therefore, future studies examining OIH should consider a multiple receptor approach is needed in order to prevent the development of OIH, which would lead to a better treatment options for pain management.

Background

Opioid analgesics typically have their greatest affinity for the mu opioid receptor (MOR). Signaling through the MOR is also attributed to the analgesic mechanism of action. The opioid analgesic, morphine, shares this affinity profile having a greater affinity for MOR (14 nM) less affinity for the kappa opioid receptor (538 nM) and lowest affinity for the delta opioid receptor (>1000 nM) (Raynor et al., 1994). Morphine like any drug has affinity for multiple receptors

and with an increased dose the probability of binding additional receptors increases. Therefore, any effects adverse or therapeutic could be occurring through opioid or non opioid receptor binding.

The receptor signaling events responsible for morphine-induced hypernociception is still unclear. However, recent studies have shown that morphine-induced hypernociception occurs in triple opioid knockout mice demonstrating this behavioral state can induced through a non-opioid receptor (Juni et al., 2007). The mechanism behind morphine's non-opioid receptor signaling ability is undetermined. However, one possible candidate is morphine 3- β -D-glucuronide (M3G), the major metabolite of morphine, which has limited affinity for all of the opioid receptors (Pasternak et al., 1987; Skarke et al., 2005) and lacks analgesic ability (Pasternak et al., 1987; Ekblom et al., 1993). In fact, M3G has been shown to be neuroexcitatory and capable of inducing nociceptive behavior (Bartlett et al., 1994; Yaksh et al., 1986; Woolf and Fitzgerald, 1981; Lewis et al., 2010). Therefore, M3G could be responsible for the non-opioid signaling taking place with morphine but the receptor that is responsible for binding M3G is unknown. Watkins and colleagues have suggested that M3G is signaling through toll-like receptor 4 (TLR4) and that signaling through TLR4 leads to M3G-induced hypernociception (Lewis et al., 2010; Hutchinson et al., 2009; Hutchinson et al., 2010). Further studies addressing this possibility are needed.

Our previous studies with morphine demonstrated that morphine treatment induced a persistent hypernociceptive state which can be transiently reversed by

the CXCR4 antagonist, AMD3100 (Wilson et al., 2011). An interaction between opioids and chemokines has previously been demonstrated by opioid agonists such as, morphine and DAMGO, increasing CXCR4 and CCR5 on monocytes and lymphocytes (Steele et al., 2003). Additionally, a number of studies from the Rogers and Adler group have demonstrated chemokine injections into the periaqueductal grey (PAG) leads to a decrease in the anti-nociceptive effects of opioids (Szabo et al., 2002; Chen et al., 2007a; b). Therefore, opioid receptor signaling could be involved in morphine-induced changes in SDF1/CXCR4 and hypernociceptive behavior.

The goal of our current study was to determine the receptor activation responsible for inducing changes in SDF1/CXCR4 signaling we previously witnessed following repeated morphine administration. Various pharmacological tools were utilized to dissect the opioid and non-opioid receptor signaling components. To test the role of opioid receptor signaling the selective MOR agonist, DAMGO, was used. Examination of non-opioid receptor signaling, M3G and morphine co-administered with the non-selective opioid antagonist, naltrexone, were assessed in their ability to induce hypernociceptive behavior and the changes in SDF1/CXCR4 signaling in the rodent DRG.

Methods

Animals. Pathogen-free, adult female Sprague-Dawley rats (150–200 g; Harlan Laboratories, Madison, WI) were housed in temperature ($23 \pm 3^{\circ}\text{C}$) and light (12-h light: 12-h dark cycle; lights on at 07:00 h) controlled rooms with standard rodent chow and water available *ad libitum*. Experiments were performed during

the light cycle. These experiments were approved by the Institutional Animal Care and Use Committee of Indiana University/Purdue University in Indianapolis. All procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health and the ethical guidelines of the International Association for the Study of Pain. All animals were randomly assigned to either treatment or control groups.

Drugs and method of administration. All drugs were freshly prepared in saline on the day of the experiment. Intraperitoneal (i.p.) injections were administered to animals following light anesthesia. For all dosing paradigms, animals received 5 once daily injections of the following drugs: DAMGO, M3G, and morphine and naltrexone. For D-Ala², N-MePhe⁴, Gly-ol-enkephalin (DAMGO) experiments, DAMGO was administered at a dose of 10 mg/kg and purchased from Sigma-Aldrich (St. Louis, MO). Morphine 3-β-D-glucuronide (M3G) was administered in doses of 1, 5, and 10 mg/kg, supplied by NIDA Drug Supply Program. For morphine and naltrexone experiments, naltrexone (10 mg/kg) was administered 30 minutes prior to morphine (10 mg/kg) injection. Four hours following the first naltrexone injection an additional naltrexone (10mg/kg) was administered. Morphine sulfate and naltrexone were purchased from NIDA Drug Supply Program and Sigma-Aldrich (St. Louis, MO), respectively. After tactile hypernociception was established, animals were given an i.p. injection of AMD3100 (10 mg/kg) five days after the last day of the dosing paradigm (PID5). AMD3100 dose was determined from previous experiments in our lab (unpublished observations, (Wilson et al., 2011)).

Tactile Behavioral assessment von Frey filaments were used to test mechanical sensitivity before, during and after cessation of DAMGO, M3G, and morphine + naltrexone administration. Prior to initial von Frey tactile testing, all rodents were habituated to testing chambers for at least two days. Animals were tested for baseline responses (BL) at least two times before undergoing the injection paradigm (5 daily doses of M3G, DAMGO, or morphine + naltrexone). Mechanical testing with von Frey filaments during dosing paradigm was limited to injection day 3 (ID3) (data not shown). Behavioral assessment on ID3 occurred 18-20 hours after the ID2 drug administration and before ID3 drug treatment. Additional behavioral assessment following drug or vehicle administration occurred on post-injection day (PID) 5, 7, and 14. All behavioral testing was performed by laboratory assistants who were blinded to the experimental conditions and unfamiliar with the experimental aims.

The incidence of foot withdrawal in response to mechanical indentation of the plantar surface of each hindpaw was measured with a von Frey filament capable of exerting forces of 10, 20, 40, 60, 80 and 120 mN. These probes exhibit a uniform tip diameter (0.2 mm) and were applied to 6 designated loci distributed over the plantar surface of the foot (Ma et al., 2003). These 6 spots are representative of the distal nerve distributions of saphenous, tibial and sural nerves (medial to lateral) in the glabrous hindpaw. During each test, the rodent was placed in a transparent plastic cage with a floor of wire with ~1×1 cm openings. The cage is elevated so that stimulation can be applied to each hind foot from beneath the rodent. The filaments were applied in order of ascending

force. Each filament was applied alternately to each foot and to each locus. The duration of each stimulus was approximately 1 s and the inter-stimulus interval was approximately 10–15 s. The incidence of foot withdrawal is expressed as a percentage of the 6 applications of each stimulus and the percentage of withdrawals was then plotted as a function of force (Bhangoo et al., 2007a; Ma et al., 2003). The von Frey withdrawal threshold was defined as the force that evoked a minimum detectable withdrawal observed on 50% of the tests given at the same force level. For cases in which none of the specific filaments used evoked withdrawals on exactly 50% of the tests, linear interpolation was used to define the threshold.

Tissue processing and immunocytochemistry for neural tissue. Lumbar (L₃–L₆) DRG tissue was collected from DAMGO, M3G, morphine co-administered with naltrexone, and naïve rodents after animals were sacrificed and transcardially-perfused with saline followed by fixative. Fixed tissue was then embedded for sectioning and processed using immunocytochemical methodologies commonly used in this lab (Bhangoo et al., 2007a, Wilson et al., 2011). Tissue sections from L₄ and L₅ were used in immunocytochemical experiments. Tissue sections were blocked with natural horse serum blocking buffer (SuperBlock® Blocking Buffer in PBS (Thermo Scientific, Rockford, IL, Natural Horse Serum (3% v/v), Triton X (0.4% v/v)). Primary antisera used was the anti-CXCR4 rat monoclonal antibody, 2B11 (1:20,000 dilution; BD Biosciences, San Jose, CA) which binds to both human and mouse CXCR4 (Forster et al., 1998; Schabath et al., 1999). CXCR4 anti-body was dissolved in

blocking buffer and incubated overnight. After primary incubation, slides were incubated in secondary antibodies (1:1,000 dilution; anti-rat made in donkey conjugated to CY3, Jackson ImmunoResearch, West Grove, PA).

Images were taken with an intensified CCD camera (Photometrics CoolSnap HQ²) coupled to a Nikon microscope (Nikon Eclipse Ti) using Nikon Elements Software (Nikon Instruments Inc., Melville, NY). Tissue sections were illuminated with a Lamda DG-4 175 W xenon lamp with an exposure time of 300 msec.

Preparation of acutely dissociated dorsal root ganglion neurons. The L1-L6 DRGs were acutely dissociated using methods described by Ma and LaMotte (Ma and LaMotte, 2005). Briefly, L1-L6 DRGs were removed from naïve and M3G treated animals four to six days following the last day of the dosing paradigm. The DRGs were treated with collagenase A and collagenase D in HBSS for 20 minutes (1 mg/ml; Roche Applied Science, Indianapolis, IN), followed by treatment with papain (30 units/ml, Worthington Biochemical, Lakewood, NJ) in HBSS containing .5 mM EDTA and cysteine at 35°C. The cells were then dissociated via mechanical trituration in culture media containing 1 mg/ml bovine serum albumin and trypsin inhibitor (1 mg/ml, Sigma, St. Louis MO). The culture media was DMEM, Ham's F12 mixture, supplemented with 10% fetal bovine serum, penicillin and streptomycin (100 ug/ml and 100 U/ml) and N2 supplement (Life Technologies). The cells were then plated on coverslips coated with poly-L lysine and laminin (1 mg/ml) and incubated for 2-3 hours before more

culture media was added to the wells. The cells were then allowed to sit undisturbed for 12–15 hours to adhere at 37°C (with 5% CO₂).

Intracellular Ca²⁺ imaging. The dissociated DRG cells were loaded with fura-2 AM (3 μ M, Molecular Probes/Invitrogen Corporation, Carlsbad CA) for 25 minutes at room temperature in a balanced sterile salt solution (BSS) [NaCl (140 mM), Hepes (10 mM), CaCl₂ (2 mM), MgCl₂ (1 mM), Glucose (10 mM), KCl (5 mM)]. The cells were rinsed with the BSS and mounted onto a chamber that was placed onto the inverted microscope. Intracellular calcium was measured by digital video microfluorometry with an intensified CCD camera (Photometrics CoolSnap HQ²) coupled to a Nikon microscope (Nikon Eclipse Ti) and Nikon Elements Software (Nikon Instruments Inc., Melville, NY). Cells were illuminated with a Lamda DG-4 175 W xenon lamp, and the excitation wavelengths of the fura-2 (340/380 nm) were selected by a filter changer. Sterile solution was applied to cells prior to chemokine application, any cells that responded to buffer alone were not used in chemokine responsive counts. Chemokines were applied directly into the coverslip bathing solution. If no response was seen within 1 minute, the chemokine was washed out. For all experiments, MCP1, SDF1, regulated upon activation, normal T cell expressed and secreted (RANTES/CCL5), and interferon-gamma-induced protein (IP10/CXCL10) were added to the cells in random order, after which capsaicin (3nM), high K⁺ (50 μ M) and ATP (3nM) were added. The chemokines used were purchased from R & D Systems (Minneapolis, MN; <1.0 endotoxin per 1 μ g of the protein by the LAL method), and all were used at a concentration of 100 nM to ensure maximal

activation (Bhangoo et al 2007a; Bhangoo et al 2007b). Chemokines were reconstituted in sterile 0.1%BSA/PBS, and aliquots were stored at -20°C. Calcium imaging traces were analyzed by two independent analyzers and only responses that were in agreement between two individuals were used in the counts.

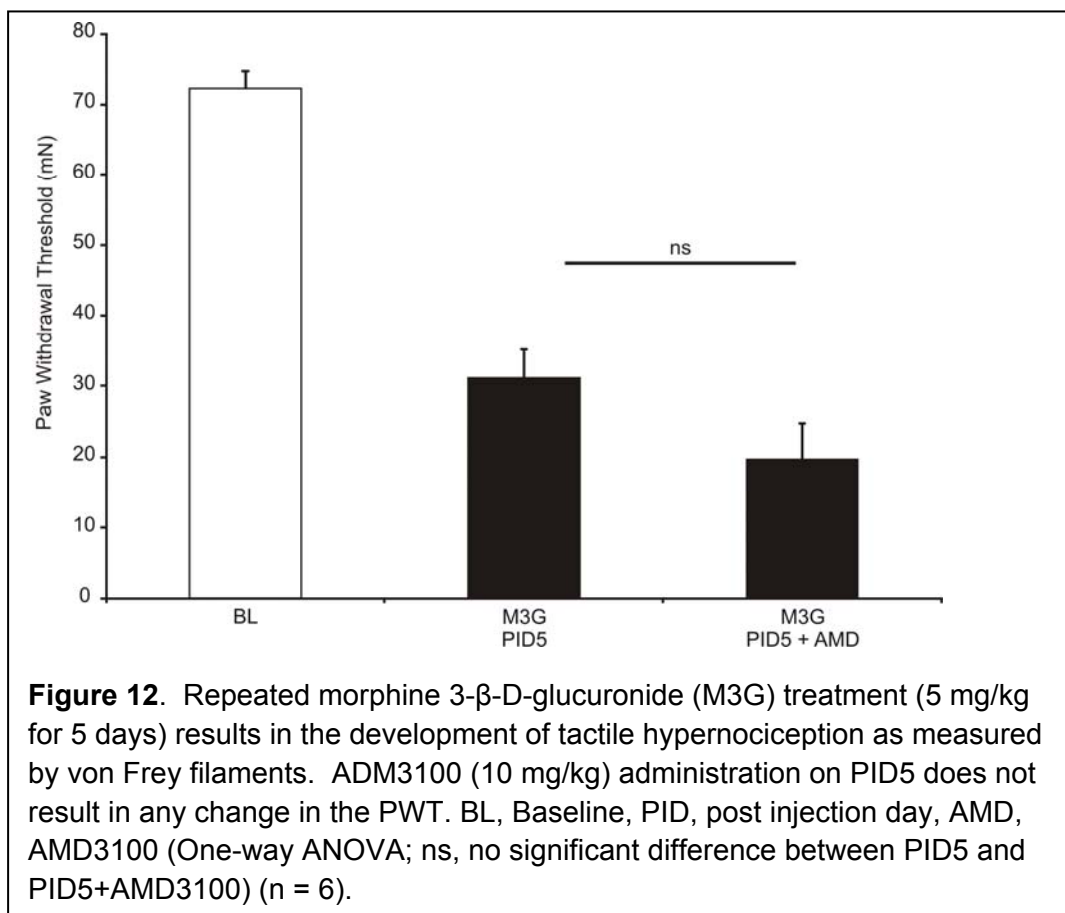
Statistics. Prism 5 (GraphPad, LaJolla, CA) was used to determine the statistical significance of differences in the mean threshold forces for foot withdrawal to punctate indentation as a function of time and between experimental groups by means of one-way analyses of variance (One-way ANOVA) followed by post hoc pairwise comparisons (Tukey method). Statistical significance was set at $p < 0.05$. GraphPad Software (LaJolla, CA) was used to determine the statistical significance of differences in calcium response among naïve and treatment groups using Chi-square test with Yates correction with $p < 0.05$ set as statistical significance.

Results

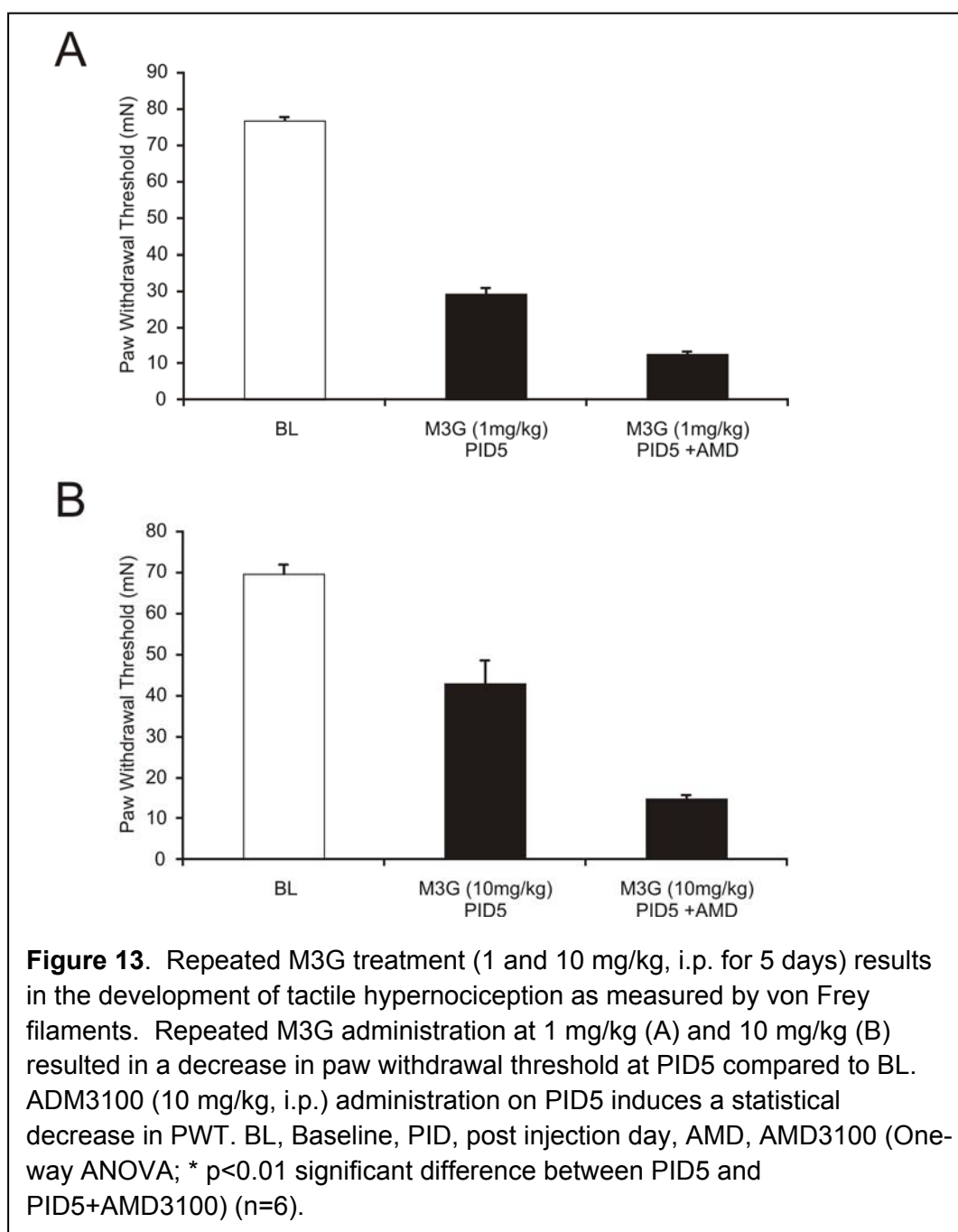
Repeated morphine 3- β -D-glucuronide (M3G) treatment leads to tactile hypernociception which is not reversed CXCR4 antagonist, AMD3100 administration

Repeated M3G administration (5 mg/kg, once a day for 5 days) was tested for its ability to induce tactile hypernociceptive as measured by von Frey filaments. In our experiments tactile behavior was assessed prior to M3G administration (BL) and five days post M3G dosing paradigm (PID5). The mean paw withdrawal threshold (PWT) was significantly decreased at PID5 when

compared to BL (72 ± 1.4 mN to 31 ± 3.0 mN; $n=6$) (**Fig. 12**). A significant decrease in PWT was also witnessed with 1 and 10 mg/kg, however, no dose dependence was witnessed (**Fig. 13**).

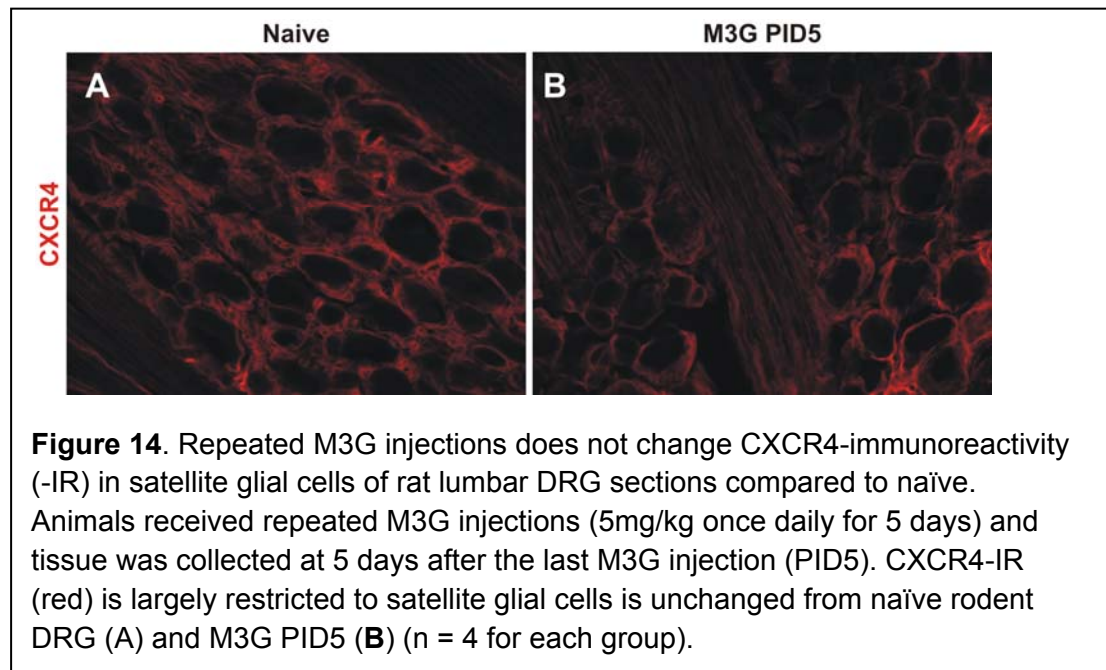


The ability of the CXCR4 antagonist, AMD3100, to reverse M3G-induced nociceptive behavior was assessed and no significant increase in PWT was observed (**Fig. 10**). Suggesting that repeated M3G administration does not lead to changes in SDF1/CXCR4 signaling.



CXCR4-immunoreactivity (ir) in rat DRG is unchanged following repeated M3G treatment

CXCR4-ir in the rat DRG is largely limited to the nonmyelinating satellite glial cells (SGCs) and a few neurons (**Fig. 14A**) in agreement with our previous findings (Wilson et al., 2011). Our previous studies demonstrated following repeated morphine administration (10 mg/kg, once a day injections for 5 days) a decrease in the CXCR4-ir at PID5. However, repeated intraperitoneal M3G injections (5 mg/kg) does not induce any change in CXCR4-ir in the rat DRG (**Fig. 14B**). The lack of changes in CXCR4-ir supports the inability of the CXCR4 antagonist, AMD3100, to reverse M3G-induced hypernociception.



Repeated morphine 3- β -D-glucuronide (M3G) does not increase functional CXCR4 receptors on DRG neurons.

To further investigate the expression of functional CXCR4 receptor, we utilized Fura-2 Ca^{2+} imaging studies in acutely dissociated lumbar DRGs derived from animals subjected to repeated M3G conditions and naive controls from previous studies. The time points used corresponded to time points used for immunohistochemical and behavioral assessment (**Figs. 12 and 14**). The acutely dissociated DRG preparations were categorized into three neuronal and non-neuronal cell types: non-capsaicin sensitive neurons (high K and ATP responsive), capsaicin sensitive neurons (capsaicin, high K, and ATP responsive), and glia (ATP responsive only). These cell response criteria were chosen strictly as an indicator of the types of cells that may be affected by the repeated M3G treatment paradigm. The tested chemokines were selected so as to activate a wide spectrum of chemokine receptors known to be expressed by neurons and non-neuronal cells (CXCR4-SDF1/CXCL12, CXCR3-IP10/CXCL10, CCR2-MCP1/CCL2, CCR5-RANTES/CCL5). The chemokine concentration used for these experiments were based on their maximally effective concentrations used in our previous observations on acutely dissociated DRGs (Bhangoo et al., 2007a; Bhangoo et al., 2007b).

Naïve acutely dissociated DRGs produced $[\text{Ca}^{2+}]_i$ changes in a small percentage of neuronal and non-neuronal populations following application of all chemokines (**Table 3**), as shown in our previous studies (Wilson et al., 2011). Repeated M3G treatment induced a significant increase in the chemokine

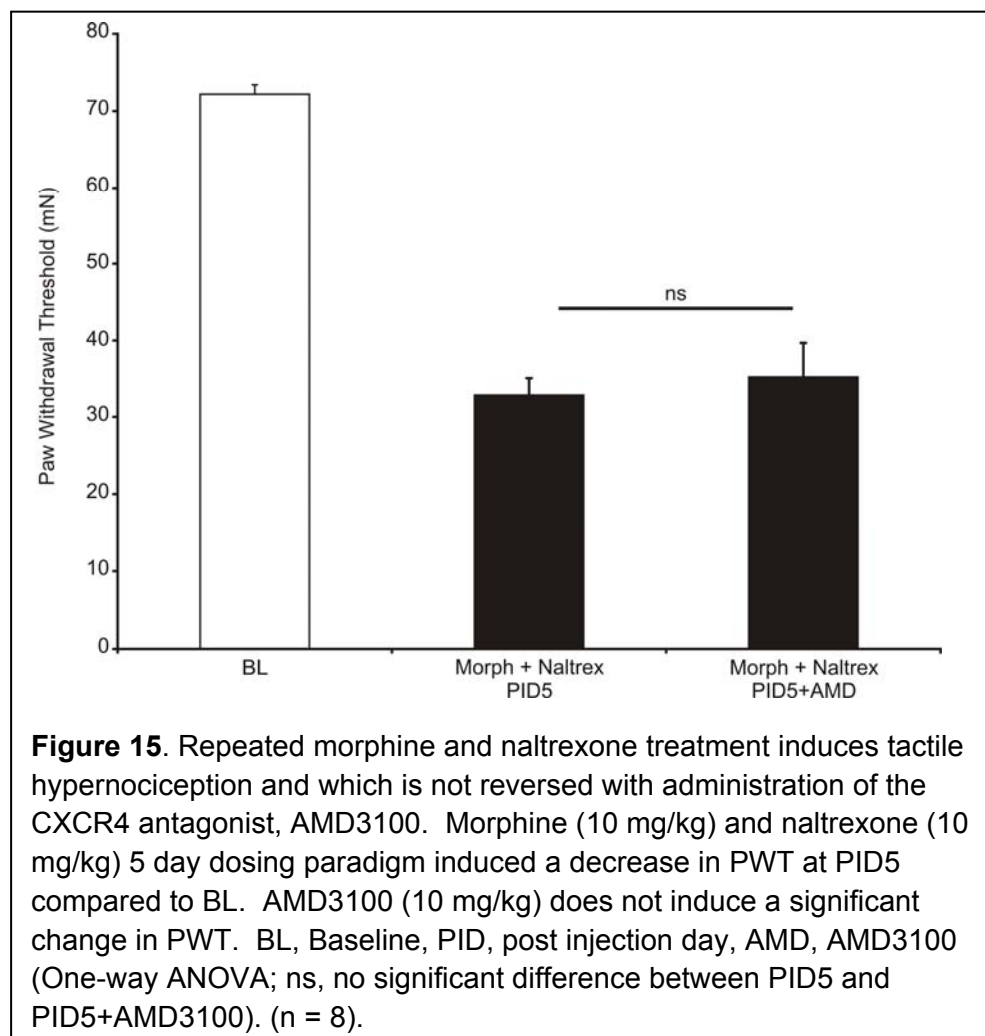
(MCP1, IP-10, and RANTES) responsiveness of non-capsaicin sensitive and capsaicin sensitive neurons (**Table 3**). Interestingly, no significant increase in SDF1 responsiveness occurred in any cell type following repeated M3G treatment (**Table 3**). Therefore, it appears there is a lack of increased functional CXCR4 receptors on DRG neurons following repeated M3G treatment in agreement with the lack of changes in CXCR4-ir and inability of CXCR4 antagonist, AMD3100 to reverse M3G-induced tactile behavior.

Naïve	Percent Positive Responding Cells			
	SDF1	IP-10	MCP1	RANTES
Non-capsaicin sensitive neuron (n=85)	7% (6/85)	4% (3/85)	4% (3/85)	14% (12/85)
Capsaicin sensitive neuron (n=72)	7% (5/72)	0% (0/72)	6% (4/72)	4% (3/72)
Glia (n=44)	21% (9/44)	2% (1/44)	9% (4/44)	21% (9/44)
M3G Treated PID4-6				
	SDF1	IP-10	MCP1	RANTES
Non-capsaicin sensitive neuron (n=74)	18% (13/74)	55%** (41/74)	28%** (21/74)	31%* (23/74)
Capsaicin sensitive neuron (n=117)	11% (13/117)	27%** (31/117)	21%** (24/117)	26%** (30/117)
Glia (n=25)	16% (4/25)	0% (0/25)	4% (1/25)	8% (2/25)

Table 3. Repeated M3G treatment **does not** induce any significant increase in the percentage of nociceptive neurons that respond to SDF1 administration as indicated by a change in intracellular calcium. Daily M3G injections (5 mg/kg for 5 days) were administered to animals. Lumbar DRG were acutely dissociated from these animals 4-6 days following the last M3G injection. Following repeated M3G administration there was a significant increase in calcium responsiveness with MCP1, IP-10, and Rantes application in capsaicin sensitive and non-capsaicin sensitive neurons. No significant increase was seen following SDF1 application in all cell types following M3G administration (** p<0.001, * p<0.05, Chi-square with Yates correction).

Repeated morphine and naltrexone treatment induces nociceptive behavior that is not reversed by CXCR4 antagonist, AMD3100, administration

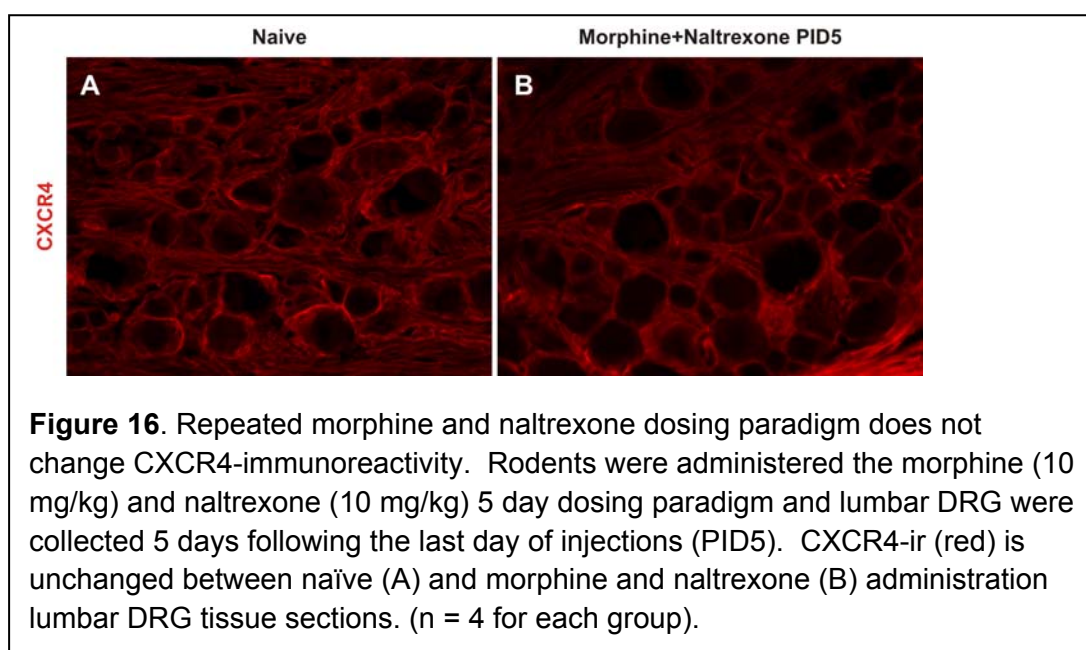
In order to ensure that M3G-induced signaling did not differ from non-opioid receptor signaling induced by morphine administration we used the non-selective opioid antagonist, naltrexone (10 mg/kg) co-administered with repeated morphine (10 mg/kg) treatment. Naltrexone was selected over naloxone because of its longer half-life (Verebey et al., 1976). Additionally, naltrexone was administered prior to morphine (10 mg/kg) injections in order to antagonize any opioid receptor activation with morphine administration and was administered four hours later to maintain naltrexone serum levels (Kim et al., 1988). The naltrexone dose of 10 mg/kg was used because of its ability to have full occupancy of MOR (Brown and Panksepp, 2009). The dosing paradigm of repeated morphine and naltrexone induced a reduction in PWT when compared to BL (72.2 ± 1.3 mN to 32.8 ± 2.2 mN; $n=8$) (**Fig. 15**).



The role of SDF1/CXCR4 signaling in this hypernociceptive behavior state was tested using the CXCR4 antagonist, AMD3100. AMD3100 was unable to reverse the morphine and naltrexone induced tactile behavioral state (**Fig. 13**). Suggesting that SDF1/CXCR4 signaling is not involved in the hypernociceptive behavior induced by morphine and naltrexone injections.

CXCR4-ir is unchanged following repeated morphine and naltrexone treatment

CXCR4-ir is localized in the SCCs in the naïve rat DRG (**Fig. 16A**) and is unchanged following repeated morphine and naltrexone treatment (**Fig. 16B**). The lack of changes in CXCR4-ir following repeated morphine and naltrexone treatment supports the inability of AMD3100 to reverse tactile behavior.

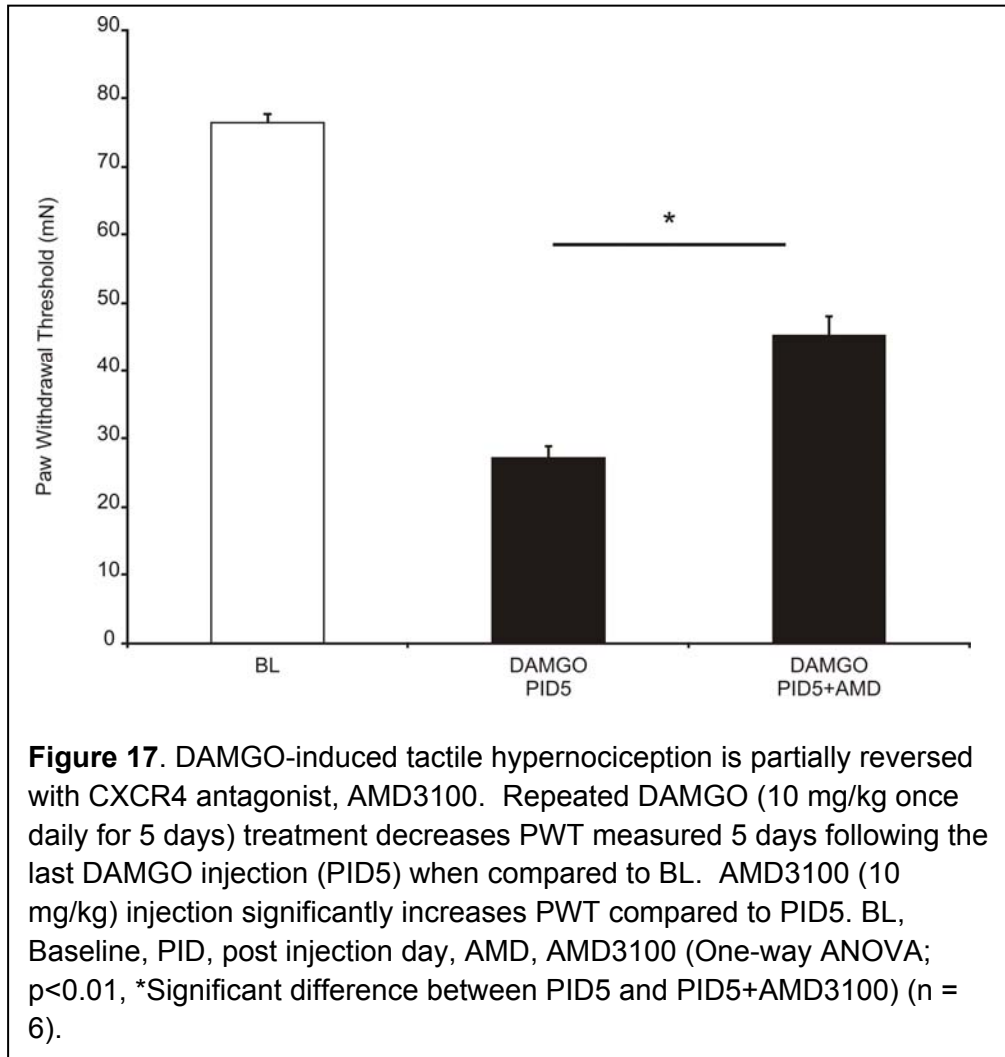


Repeated DAMGO injections induce hypernociceptive behavior that is partially reversed by administration of the CXCR4 antagonist, AMD3100

In order to determine the role of MOR activation in changes in SDF1/CXCR4 signaling witnessed following repeated morphine treatment we employed the selective MOR agonist, DAMGO. DAMGO has similar half-life pharmacokinetics to morphine, thus the same dosing paradigm was used (once a day injections for 5 days) (Szeto et al., 2001). DAMGO is unable to cross the

blood-brain-barrier and therefore is rarely administered systemically, however, DAMGO (10 mg/kg, ip) injections have been shown to be anti-nociceptive to guarding behavior and was employed in our studies (Craft et al., 1995). DAMGO injections (10 mg/kg, once a day for five days) induced a hypernociceptive state through a reduction in PWT at PID5 (27.2 ± 1.7 mN; $n=6$) when compared to BL (76.5 ± 1.3 mN) (**Fig. 17**).

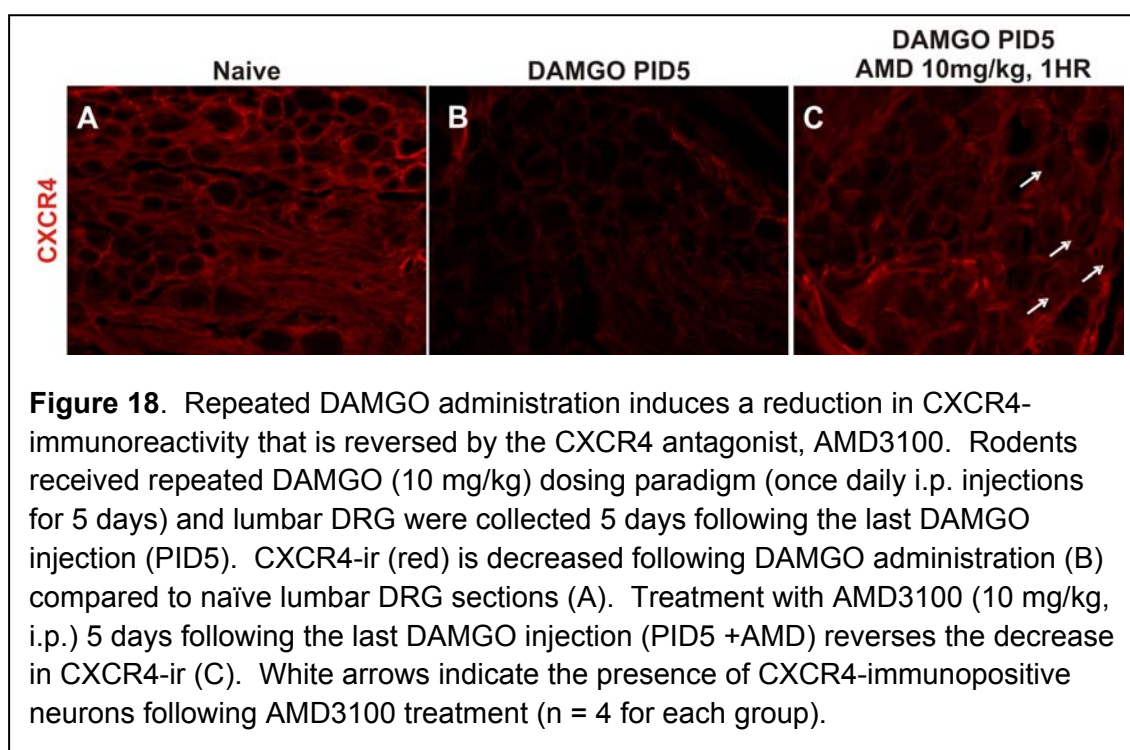
To test the role of changes in SDF1/CXCR4 signaling in DAMGO-induced hypernociception, AMD3100 was administered five days following the last DAMGO injection (PID5) and was able to significantly increase the PWT when compared to PID5 prior to AMD3100 administration (27.2 ± 1.7 mN to 45.2 ± 3.0 mN; $n=6$) (**Fig. 17**).



Decrease in CXCR4-ir following repeated DAMGO administration is transiently reversed with administration of CXCR4 antagonist, AMD3100

CXCR4-ir in the naïve rat lumbar DRG is localized primarily to the satellite glial cells (**Fig. 18A**) and decreases following repeated DAMGO administration (**Fig. 18B**). Following intraperitoneal administration of the CXCR4 antagonist, AMD3100 (10 mg/kg) and sacrificing animals one hour post AMD3100 injection, CXCR4-ir returns to near naïve levels (**Fig. 18C**). CXCR4-ir positive neurons

(white arrows) are also evident in DAMGO treated lumbar DRG tissue sections following AMD3100 administration (**Fig. 16C**). These findings mimic the changes in CXCR4-ir that we observed in our previous studies following repeated morphine administration (Wilson et al., 2011). Additionally, the presence of CXCR4-immunopositive neurons supports the ability of AMD3100 to partially reverse DAMGO-induced tactile hypernociception.



Discussion

Our results demonstrate that repeated M3G, DAMGO, and morphine co-administered with naltrexone are all capable of inducing nociceptive tactile behavior. However, only repeated DAMGO administration induces changes in SDF1/CXCR4 signaling, similar to those reported in our previous findings with repeated morphine treatment (Wilson et al., 2011). This change in SDF1/CXCR4

signaling is evident by the decrease in CXCR4-ir following repeated DAMGO administration that is reversible by the administration of AMD3100, CXCR4 antagonist. Additionally, AMD3100 is able to partially reverse DAMGO-induced tactile nociceptive behavior.

The results of our current study suggest that morphine-induced nociceptive behavior is induced by two mechanisms, opioid and non-opioid receptor signaling. MOR signaling through repeated DAMGO administration is capable of inducing a decrease in paw withdrawal threshold. Similarly, compounds that prevent or have limited opioid receptor signaling capability, M3G and morphine co-administered with naltrexone, are also capable of inducing nociceptive behavior. To our knowledge, we are the first to demonstrate the ability of systemic M3G administration to induce nociceptive behavior. Interestingly, although DAMGO, M3G, and morphine + naltrexone all induced nociceptive behavior the behavior is not as long lasting (data not shown) as previously seen with repeated morphine administration which persisted for at least 28 days (Wilson et al., 2011). Therefore, the mechanism needed to maintain the nociceptive behavior over time must require the signaling events from both opioid and non-opioid receptors that would be occurring from morphine treatment.

Our findings indicate that the changes in SDF1/CXCR4 signaling are induced by MOR activation through DAMGO administration. These findings are in agreement with previous studies conducted in immune cells (Happel et al., 2008). These studies demonstrated that increased CXCR4 expression on

leukocytes is induced with DAMGO administration and is naloxone reversible (Happel et al., 2008). Therefore, while nociceptive behavior is capable of being produced through opioid and non-opioid receptor signaling, the SDF1/CXCR4 signaling component of this behavior is dependent on MOR activation.

Treatment with the CXCR4 antagonist, AMD3100, only partially reversed the nociceptive behavior following repeated DAMGO administration. However, following repeated morphine administration AMD3100 was capable of fully reversing the nociceptive behavior (Wilson et al., 2011). One possible explanation behind these results is that DAMGO is unable to cross the blood brain barrier and therefore any signaling it induces would be occurring in the peripheral nervous system. While CXCR4 expression is limited in the spinal cord, central canal and neuroepithelium, (personal observation), there is a great deal of opioid receptor expression in the spinal cord (Kuhar et al., 1973; Pert et al., 1976; Lamotte et al., 1976; Atweh and Kuhar, 1977; Fields et al., 1980; Ninkovic et al., 1982). Therefore, perhaps opioid signaling in the CNS is needed to perpetuate the changes in SDF1/CXCR4 signaling previously seen with morphine administration.

An alternative explanation is that other opioid receptor signaling, DOR and KOR, is needed to potentiate changes in SDF1/CXCR4 signaling. As noted previously morphine has affinity for all three opioid receptors and therefore could be signaling through KOR or DOR in order to induce changes. DOR signaling could be involved in our previous findings with morphine, Gendron and colleagues demonstrated that similar repeated morphine treatment induces

increased DOR expression on the DRG cell membrane (Gendron et al., 2006). DOR in the naive rat DRG is located in vesicles at the cell membrane and following repeated morphine administration DOR are inserted into the cell membrane (Gendron et al., 2006). DOR would be available for signaling and have potential in potentiating changes in SDF1/CXCR4 signaling. Although, studies conducted with a selective delta agonist such as, [D-Pen^{2,5}] Enkephalin , [D-Pen²,D-Pen⁵] Enkephalin (DPDPE), could give a false negative result because although DORs are not responsible for the initial signaling events with morphine, DOR signaling events could occur with later morphine administration or signaling events following the cessation of morphine administration. Morphine signaling through the KOR should also be explored. KOR's role in the changes in SDF1/CXCR4 signaling is questionable. The selective KOR agonist, U-50488, inhibits LPS and HIV-tat-induced increases in cytokine levels in a number of cell types (Zhang and Rogers, 2000; Belkowski et al., 1995; Alicea et al., 1996; Neudeck et al., 2003; Sheng et al., 2003). However, one study conducted by the Rogers lab demonstrated that KOR activation is capable of increasing the expression of CCR2 mRNA in thymocytes treated with LPS (Zhang and Rogers, 2000). Most studies point to an opposing effect between MOR and KOR in cytokine expression and it would be interesting to see if this holds true in the DRG. Furthermore, most of these studies were conducted in activated cells; the ability of KOR agonist to induce changes in cells at a resting state should also be explored.

In conclusion, opioid and non-opioid receptor activation is responsible for the induction of OIH. However, changes in SDF1/CXCR4 signaling found in our previous studies with repeated morphine treatment (Wilson et al., 2011) are dependent on mu opioid receptor activation. These findings are interesting because the analgesic ability of opioids is also thought to result from mu opioid receptor activation. Thus, new analgesic treatment options should take into account both the beneficial and detrimental effects of opioid receptor activation and attempt to develop treatments to address these issues.

CHAPTER FOUR

DISCUSSION

Chronic pain affects many individuals decreasing their quality of life. The treatment options for controlling these individuals pain is limited and often times ineffective. The mechanism of action behind most current therapies is unknown. Much of this stems from a lack of understanding of the diverse causes of chronic pain and all possible targets for drug treatments. A better mechanistic understanding of current treatment options, such as opioid analgesics, would allow for the development of new, more effective pain therapeutics and was therefore the goal of this project. By providing a better understanding of opioid agonist effects and receptor targets will allow for the development of more effective opioid analgesics or alternative therapeutics.

Overview of Results

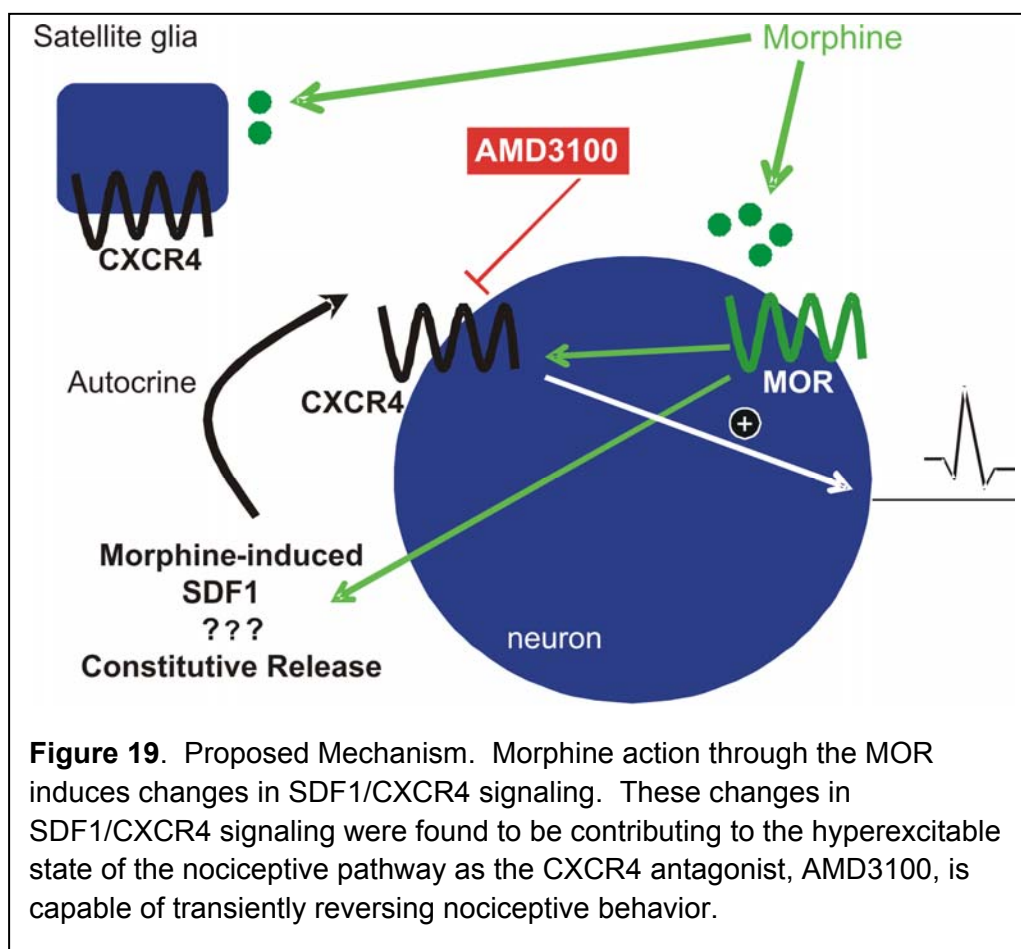
The purpose of this project was to examine the role of SDF1/CXCR4 signaling in opioid induced hypernociception and to identify receptor signaling events responsible for these changes. The first part of the project was to first establish a morphine dosing paradigm that would induce long lasting changes in nociceptive behavior. The repeated morphine paradigm (once daily 10 mg/kg i.p. injections for 5 days) induced tactile nociceptive behavior that persisted for at

least 28 days following the cessation of morphine administration. No change in thermal behavior was witnessed, which is in contrast to other dosing protocols of opioids of twice daily injections or intrathecal administration (Mao et al., 1994; Raghavendra et al., 2004). The systemic morphine dosing paradigm was selected because it better mimics the route of administration in the clinical setting.

Following the establishment of long lasting nociceptive behavior we found this morphine dosing paradigm induces changes in SDF1/CXCR4 signaling in the DRG. The decrease and return of CXCR4-ir in rodent DRG tissue sections witnessed coincides with the development and recovery of OIH. Furthermore, administration of the CXCR4 antagonist, AMD3100, following the cessation of morphine administration transiently reversed the established tactile nociceptive behavior. Indicating the central role that SDF1/CXCR4 signaling is playing in OIH. Administration of AMD3100 revealed the tonic activation process taking place between SDF1 and CXCR4 in the rodent DRG through the ability of AMD3100 to reverse the decrease in CXCR4-immunoreactivity. Release studies in F11 cells provided evidence of SDF1's constitutive release characteristics further validating the tonic activation between SDF1 and CXCR4 in the rodent DRG following morphine administration.

The second part of this project focused on investigating the receptor signaling events responsible for the observed morphine-induced tactile behavior and changes in SDF1/CXCR4 signaling. Tactile nociception was found to be

induced by both non-opioid receptor signaling compounds, M3G and morphine co-administered with the non selective opioid antagonist, naltrexone, and the selective MOR agonist, DAMGO. These results suggest that OIH witnessed with repeated morphine administration is induced by opioid and non-opioid receptor signaling events. However, changes in SDF1/CXCR4 signaling in the rodent DRG were found to be dependent on MOR activation, as shown by repeated DAMGO administration.



Another notable finding from this project was revealed in the calcium imaging studies. All chemokines examined (SDF1, RANTES, MCP, IP-10) had a significant increase in responsive neurons following morphine administration. Following M3G administration all chemokines except for SDF1 had a significant increase in the percentage of responsive neurons. These results indicate that signaling from other chemokine and receptor pairings are upregulated in both morphine and M3G tactile nociceptive behavior states. Further investigation into the role of other chemokines in each of those tactile behavior states is warranted. A preliminary study evaluating MCP1/CCR2's role in OIH presented in **Appendix B**. CCR2 antagonism was incapable of reversing morphine induced tactile behavior (**Fig. 18**). These results were surprising following calcium imaging studies revealing an increase in MCP1 responsive neurons, indicative of increased functional CCR2. The calcium imaging and behavioral studies were conducted at the same time point following repeated morphine treatment. However, an increase in CCR2 positive neurons is not indicative of increased signaling. The absence of a CCR2 ligand in the DRG would prevent increased CCR2 signaling, thereby preventing the ability of CCR2 antagonism to be successful at reversing any established nociceptive behavior. Therefore, these results indicate the importance of multiple techniques in demonstrating changes in chemokine signaling. Changes in receptor or ligand expression are not sufficient in demonstrating a central component of any physiological state. Additionally, changes in MCP1/CCR2 or the other chemokines could require a

longer time to develop. Further studies investigating the time course of the development of chemokine changes and the role of the other chemokines in OIH is warranted.

Possible non-opioid receptor targets

Our results using non-opioid signaling compounds to induce tactile nociceptive behavior were not surprising. Previous studies in triple opioid knock-out mice revealed morphine's retained ability to induce nociceptive behavior (Juni et al., 2007). Additionally, intrathecal administration of M3G induces nociceptive behavior (Lewis et al., 2010). However, studies investigating the receptor or receptors responsible for these non-opioid signaling compounds effect are largely limited. Watkins and colleagues propose that TLR4 is the receptor responsible for these changes (Lewis et al., 2010; Hutchinson et al., 2009; Hutchinson et al., 2010). My data presented in **Appendix A** demonstrate a significant increase in the number of TLR4 responsive neurons following morphine and M3G administration (**Table 3**). Additionally, immunocytochemistry studies reveal the presence of TLR4-ir positive neurons in the lumbar DRGs of naïve, morphine, and M3G treated animals (**Fig 17A**). Repeated morphine (10 mg/kg) and M3G (10 mg/kg) both induced a significant increase in TLR4-positive neurons (**Fig 17B**). The presence of TLR4-positive neurons show the possibility of TLR4 signaling to be occurring in the rodent DRG. However, the binding ability of morphine and M3G to TLR4 and the ligand responsible for increased TLR4 signaling within the DRG needs to be further examined.

An alternative explanation for the increased LPS responsiveness following morphine and M3G could be linked to increased neuronal CXCR4 expression. CXCR4 has been proposed to be part of the LPS “sensing apparatus” (Triantafilou et al., 2008). These studies conducted in CXCR4 transfected HEK cells demonstrate that both SDF1 and LPS are capable of inducing changes in IL-6 levels, p-MAPK, and inducing chemotaxis (Triantafilou et al., 2008). Indeed LPS competitively inhibit the binding of SDF1 in CXCR4 transfected HEK cells. Therefore, the increases in LPS calcium responsiveness could be occurring from the increased CXCR4 neuronal expression. However, the presence of increased LPS responsiveness following M3G administration, conditions where SDF1 responsive cells are not significantly increased suggests that a separate LPS signaling receptor is required. The specific receptor responsible for non-opioid signaling has yet to be determined and future studies investigating this topic would serve to provide better more selective opioid analgesics.

CXCR7

With the discovery of SDF1’s capability of binding to not only CXCR4 but also CXCR7 raises the question of CXCR7’s role in SDF1 signaling (Balabanian et al., 2005). CXCR7 was initially described as a scavenger receptor (Boldajipour et al., 2008; Dambly-Chaudiere et al., 2007); more recent interactions describe CXCR7 as possibly moderating the response of CXCR4 to SDF1 by internalizing the ligand (Zabel et al., 2009). Although CXCR7 does not elicit activation of G-protein signaling pathways, it does activate MAP kinases

through β -arrestin (Rajagopal et al., 2010) and is therefore capable of altering the excitation state of cells. Additionally, like SDF1, AMD3100 was previously known to selectively block CXCR4 signaling. Recent studies have demonstrated that SDF1 and AMD3100 both stimulate arrestin recruitment to CXCR7, and AMD3100 may act as an allosteric agonist of CXCR7 (Kalatskaya et al., 2009). If so, changes in CXCR7 neuronal expression in the DRG following M3G administration (1 and 10 mg/kg, i.p.) could explain the significant decrease in paw withdrawal threshold following AMD3100 administration in these animals (**Fig. 11**). However, the presence of CXCR7 in the rodent DRG is unknown and future studies would need to be conducted to investigate this possible signaling event. These results would challenge the effectiveness of AMD3100 in states where both CXCR4 and CXCR7 are both present.

Given the possibility that CXCR7 signaling via β -arrestin may contribute to SDF1-mediated cellular functions following repeated morphine treatment, it is important to know the neuronal distribution of CXCR7 in the rodent. The lack of reliable CXCR7 antibodies thus far has prevented the ability of anatomical localization of this receptor in the rat DRG. However, CXCR7-GFP mice developed in Dr. Richard Miller's lab increase the expression CXCR7 in both sensory neurons and SGCs following LPS treatment (unpublished observations). Merely the observation of neuronal CXCR7, regardless of the conditions, provides us with a basis to speculate about possible SDF-1 influences on CXCR7 in the DRG, including neuronal excitability. One possibility that has been

described in T cell lymphocytes is that CXCR7 forms heterodimers with CXCR4 which may alter CXCR4 $G_{\alpha i}$ -protein activation and subsequent calcium fluxes (Levoye et al., 2009). Additionally, CXCR7 could be working in our model of repeated morphine treatment to further regulate the levels of SDF1 in the DRG. Clearly, the role of CXCR7 in neurobiology still needs to be determined and its role in nociceptive states is an area requiring further investigation.

SDF1/CXCR4 Changes in Satellite Glial Cells

Changes in SDF1/CXCR4 signaling in sensory neurons directly affect the nociceptive behavior witnessed in OIH. However, the reduction in CXCR4-immunoreactivity in the satellite glial cells (SGC) following repeated morphine and DAMGO administration demonstrate increased SDF1/CXCR4 signaling in SGCs as well. While SDF1/CXCR4 signaling in SGCs would be indirect the signaling event could still be contributing to OIH. A potential mechanism of SDF1/CXCR4 signaling in SGCs could be occurring through the release of pro-inflammatory mediators into the milieu of the DRG thereby, indirectly affecting the excitatory state of sensory neurons. Recent evidence has emerged that implicates SDF1 induction of the pro-inflammatory cytokine, IL-6 (Tang et al., 2008) and may also control expression of other chemokines such as neuronal fractalkine (Cook et al., 2010). SDF1's ability to induce IL-1 β would further contribute through the paracrine interactions within the rat DRG. IL-1 β can be released from satellite glial cells whereupon it acts on nearby small-diameter sensory neurons bearing interleukin type I receptor (IL-1RI) (Takeda et al., 2008).

In turn, IL-1 β signaling significantly increases the spontaneous neuronal activity in A δ mechanosensitive neurons and may be important in hypernociception (Takeda et al., 2008). Furthermore, it is commonplace that cytokines induce chemokine promoter activation and increase secretion of chemokines during inflammation, which could be responsible for the maintenance of the observed OIH behavior.

The satellite glial cells in the rodent DRG could have an alternative role. In the naïve rodent DRG the majority of CXCR4 expression is located on SGCs. However, the role that CXCR4 expression on SGCs is playing in OIH was not addressed in these studies. One possibility is that CXCR4 on SGCs is acting as a “sink” for released SDF1, serving to buffer any SDF1 that is released within the DRG or SDF1 from the systemic circulation. In support of this theory, calcium imaging studies conducted on acutely dissociated DRGs, only 20 percent of glial cells have a calcium response to SDF1 in both naïve and morphine treated conditions (Wilson et al., 2011). This suggests SDF1/CXCR4 signaling in SCCs occurs through a different mechanism. SDF1 is unable to bind the non-signaling chemokine receptors, D6 and DARC, and was shown to be alternatively internalized by CXCR4 to maintain the homeostatic levels of SDF1 (Dar et al. 2005). This theory would coincide with other studies that have shown the ability of SGCs to take up molecules from the milieu of the DRG (Kumamoto et al., 1986; Schlaepfer, 1969). This mechanism supports the need for CXCR4 on

SGCs to act as a buffering system for circulating SDF, preventing any unnecessary CXCR4 activation by circulating levels of SDF1.

Opioid-induced changes in PNS chemokine/ receptor expression

The mechanisms underlying the effects of chronic opioid treatment on chemokine/receptor expression are not fully understood, but is based on evidence that opioids stimulate the production and release of cytokines in many cell types including leukocytes, keratinocytes, and glial cells (Volk et al., 2004; Messmer et al., 2006; Wang et al., 2007; Liang et al., 2008; Johnston et al., 2004; Tai et al., 2006). It is well known that cellular activation by these cytokines initiate signaling cascades that eventually lead to the expression of chemokines/receptor. For example, upregulation of CXCR4 following DAMGO administration is thought to be dependent on the naloxone-reversible increases in TGF- β production (Chao et al., 1992; Steele et al., 2003; Happel et al., 2008). Chronic morphine treatment also increases the expression of the chemokine receptors, CCR2 and CCR5, in human astrocytes and CCL2 in human neurons via largely unknown mechanisms (Mahajan et al., 2005; Rock et al., 2006). Taken together, this evidence suggests that chronic opioid treatment directly or indirectly can lead to upregulation of chemokine signaling in leukocytes, neurons and glial cells.

Despite evidence of direct or indirect, opioid-induced transcription factor regulation in variety of cells types, little work has been conducted to investigate transcriptional control of chemokine/receptor expression in either glial cells or

neurons. The promoter regions of a number of chemokine/receptors were analyzed by Hosung Jung in the Miller lab and candidate transcription factors were identified which include NFAT, NF-KB, CREB, and C/EB. The Miller lab further investigated the transcriptional control of chemokines in F11 cells and acutely dissociated sensory neurons subjected to pathological circumstances of chronic excitability (Jung and Miller, 2008). Jung and Miller (2008) demonstrated that DRG-like F11 cells expression of the chemokine receptors, CCR2 and CCR5 is depended on nuclear factor of activated T cells (NFAT) activation, whereas chemokine receptor, CXCR4, was not. With this outlined information known about the transcriptional control of chemokines and their receptors which of these transcription factors is induced by opioid treatment needs to be examined.

Opioid receptor control of transcription factors has been explored in a number of different cell types. Neurons, macrophages, and immune cells treated with MOR agonists induce the activation and increased DNA binding of the transcription factors NF-KB, CREB, and AP-1 (Hou et al., 1996; Roy et al., 1998; Bilecki et al., 2004; Happel et al., 2010). Furthermore, DAMGO treatment increases CCL2 expression in polymorphonuclear cells which is dependent on NF-KB activity (Happel et al., 2010). Selectively targeting the downstream transcriptional regulation of chemokines and receptors following opioid administration could serve to prevent some of the detrimental effects of opioid analgesic and improve their therapeutic effects.

Chemokines Role in Inflamed vs. Un-inflamed Tissue

Cells of the immune system utilize the chemoattractant gradient of chemokines in order to localize to sites of injury. Stein and colleagues were able to demonstrate a secondary role in which the chemokine CXCL2/3 is able to stimulate the release of opioids from opioid-containing immune cells such as leukocytes (Rittner et al., 2006). This chemokine-induced release of endogenous opioids is beneficial and analgesic when chemokines are injected into the hindpaw in a model of inflammatory pain (Rittner et al., 2006). This is in direct contrast to the nociceptive effects of chemokines injected into an un-inflamed hind paw (Oh et al., 2001). In the un-inflamed hindpaw the chemokines have no opioid containing immune cells to act on and are in this instance detrimental because chemokines are capable of activating sensory neurons (White et al., 2005).

SDF1 was also tested in these studies and was unable to induce the release of endogenous opioids from leukocytes expressing CXCR4 (Rittner et al., 2006). However, SDF1 application did not lead to as substantial of a calcium response at the concentration used and the concentration of SDF1 needed to induce a significant chemotactic response was 100 fold greater than that required for CXCL2/3 (Rittner et al., 2006). Additionally, while SDF1 was not found to be analgesic in these studies SDF1 did not induce a nociceptive response (Rittner et al., 2006), which differs from the findings of SDF1 in an un-

inflamed paw (Oh et al., 2001). Suggesting a greater concentration of SDF1 might be needed for the same response seen with CXCL2/3.

Findings from our lab and others demonstrate that opioid administration from both *in vitro* and *in vivo* studies act to increase the expression of both chemokines and their receptors on cells of the immune and nervous system (Steele et al., 2003; Miyagi et al., 2000; Rock et al., 2006; Mahajan et al., 2005; El-Hage et al., 2006; Wilson et al., 2011). It can be theorized that opioid-induced increases in chemokine/receptors expression would be beneficial in inflammatory pain states of which opioids are often employed. In these conditions opioids would act to increase the expression of chemokines/receptors and would thereby increase the infiltration of immune cells and increase the release of endogenous opioids at the site of injury. Opioid usage in non-inflammatory pain states would increase the expression of chemokines/receptors however; the absence of opioid-containing immune cells for the chemokines to act upon would be disadvantageous. Without the presence of opioid containing immune cells, the chemokines would act directly on the sensory neurons, inducing a hyperexcitable state. This explanation could explain why OIH in the clinical setting is reported at a site separate from the injury. At the injury site the opioid-induced increase in chemokine/receptor expression would be analgesic inducing an increase in the release of endogenous opioids. However, following the systemic administration of opioids locations away from the site of injury would also have increased

expression of chemokine/receptor, but the lack of infiltrated immune cells at these sites would lead to a hypernociceptive state.

If this theory holds true future use of opioids in pain treatment should consider the use of locally applied opioids. This approach would allow for the beneficial effectiveness of opioids, acting directly on sensory neurons and inducing increased expression of chemokines/receptors locally, thereby increasing the release of endogenous opioids and recruitment of other immune cells needed for healing. Furthermore, local opioid treatment would prevent the increase in chemokine signaling at sites separate from the injury site and avoid the hypernociceptive state that can be induced from systemic opioid administration. The analgesic ability of locally applied morphine has been demonstrated in clinical studies following knee surgery and chronically inflamed tooth pain (Dionne et al., 2001; Likar et al., 1999; Stein et al., 1991). In two of the studies locally applied morphine had a higher analgesic effect than systemic morphine (Dionne et al., 2001; Stein et al., 1991). An additional benefit of local application of opioids is the avoidance of a number of problematic side effects of opioids such as, respiratory depression and urinary retention.

APPENDIX A:
TLR4 DISTRIBUTION IN DORSAL ROOT GANGLION AND LPS
RESPONSIVE CELL IN ACUTELY DISSOCIATED DORSAL ROOT
GANGLION

Methods

Animals. Pathogen-free, adult female Sprague-Dawley rats (150–200 g; Harlan Laboratories, Madison, WI) were housed in temperature ($23 \pm 3^{\circ}\text{C}$) and light (12-h light: 12-h dark cycle; lights on at 07:00 h) controlled rooms with standard rodent chow and water available *ad libitum*. Experiments were performed during the light cycle. These experiments were approved by the Institutional Animal Care and Use Committee of Indiana University/Purdue University in Indianapolis. All procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health and the ethical guidelines of the International Association for the Study of Pain. All animals were randomly assigned to either treatment or control groups.

Drugs and method of administration. All drugs were freshly prepared in saline on the day of the experiment. Intraperitoneal (i.p.) injections were administered to animals following light anesthesia. For all dosing paradigms, animals received 5 once daily injections of the following drugs: M3G (1, 5, and 10 mg/kg) and morphine sulphate (10 mg/kg). Morphine 3- β -D-glucuronide (M3G) and morphine sulfate were supplied by NIDA Drug Supply Program.

Tissue processing and immunocytochemistry for neural tissue. Morphine, M3G or naïve control treatments rats' lumbar (L₃-L₆) DRG tissue was collected after animals were sacrificed and transcardially-perfused with saline followed by fixative. Fixed tissue was then embedded for sectioning and processed using immunocytochemical methodologies commonly used in this lab (Bhangoo et al.,

2007a). Tissue sections from L₄ and L₅ were used in immunocytochemical experiments. Primary antisera used was the anti-TLR4 goat M16 extracellular monoclonal antibody, (1:200 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA). After primary incubation, slides were incubated in secondary antibodies (anti-goat made in horse conjugated to CY3, Jackson ImmunoResearch, West Grove, PA). Positive control immunocytochemistry staining was conducted in rat spleen sections. Specific labeling of white pulp was observed.

Cell Counts. Images were taken with an intensified CCD camera (Photometrics CoolSnap HQ²) coupled to a Nikon microscope (Nikon Eclipse Ti) using Nikon Elements Software (Nikon Instruments Inc., Melville, NY). Tissue sections were illuminated with a Lamda DG-4 175 W xenon lamp. Within elements software each images maximum threshold was set between 8000 and 8500. Total cell counts for each section were taken using the grid function to aide in total cell count. TLR4 positive cell counts were conducted using Image Pro Software (Media Cybernetics, Bethesda, MD). The following parameters were used for cell counts: Intensity range (40-255), smoothness (20), measurement window size (10µM-∞). Images fluorescent artifacts such as axons and cell debris were unselected so that these were not used in cell counts. TLR4 cell counts were taken from each tissue section image and combined for each treatment group.

Preparation of acutely dissociated dorsal root ganglion neurons. The L1-L6 DRGs were acutely dissociated using methods described by Ma and LaMotte (Ma and LaMotte, 2005). Briefly, L1-L6 DRGs were removed from naïve, M3G,

and morphine-treated animals four to six days following the last day of the dosing paradigm. The DRGs were treated with collagenase A and collagenase D in HBSS for 20 minutes (1 mg/ml; Roche Applied Science, Indianapolis, IN), followed by treatment with papain (30 units/ml, Worthington Biochemical, Lakewood, NJ) in HBSS containing .5 mM EDTA and cysteine at 35°C. The cells were then dissociated via mechanical trituration in culture media containing 1 mg/ml bovine serum albumin and trypsin inhibitor (1 mg/ml, Sigma, St. Louis MO). The culture media was DMEM, Ham's F12 mixture, supplemented with 10% fetal bovine serum, penicillin and streptomycin (100 ug/ml and 100 U/ml) and N2 (Life Technologies). The cells were then plated on coverslips coated with poly-L lysine and laminin (1 mg/ml) and incubated for 2-3 hours before more culture media was added to the wells. The cells were then allowed to sit undisturbed for 12–15 hours to adhere at 37°C (with 5% CO₂).

Intracellular Ca²⁺ imaging. The dissociated DRG cells were loaded with fura-2 AM (3 uM, Molecular Probes/Invitrogen Corporation, Carlsbad CA) for 25 minutes at room temperature in a balanced sterile salt solution (BSS) [NaCl (140 mM), Hepes (10 mM), CaCl₂ (2 mM), MgCl₂ (1 mM), Glucose (10 mM), KCl (5 mM)]. The cells were rinsed with the BSS and mounted onto a chamber that was placed onto the inverted microscope. Intracellular calcium was measured by digital video microfluorometry with an intensified CCD camera (Photometrics CoolSnap HQ²) coupled to a Nikon microscope (Nikon Eclipse Ti) and Nikon Elements Software (Nikon Instruments Inc., Melville, NY). Cells were illuminated

with a Lamda DG-4 175 W xenon lamp, and the excitation wavelengths of the fura-2 (340/380 nm) were selected by a filter changer. Sterile solution was applied to cells prior to chemokine application, any cells that responded to buffer alone were not used in chemokine responsive counts. Chemokines were applied directly into the coverslip bathing solution. If no response was seen within 1 minute, the chemokine was washed out. For all experiments, MCP1, SDF1, regulated upon activation, normal T cell expressed and secreted (RANTES/CCL5), and interferon-gamma-induced protein (IP10/CXCL10) were added to the cells in random order. Following chemokine application LPS (1 μ g/mL) was applied to coverslip, after which capsaicin (3nM), high K⁺ (50 μ M) and ATP (3nM) were added. The chemokines used were purchased from R & D Systems (Minneapolis, MN; <1.0 endotoxin per 1 μ g of the protein by the LAL method), and all were used at a concentration of 100 nM to ensure maximal activation (Bhangoo et al 2007a; Bhangoo et al 2007b). Chemokines and LPS were reconstituted in sterile 0.1%BSA/PBS, and aliquots were stored at -20°C. Calcium imaging traces were analyzed by two independent analyzers and only responses that were in agreement between two individuals were used in the counts.

Statistics. GraphPad Software (LaJolla, CA) was used to determine the statistical significance of differences in calcium response and TLR4-positive neurons among naïve and treatment groups using Chi-square test with Yates correction with $p < 0.05$ set as statistical significance.

Results

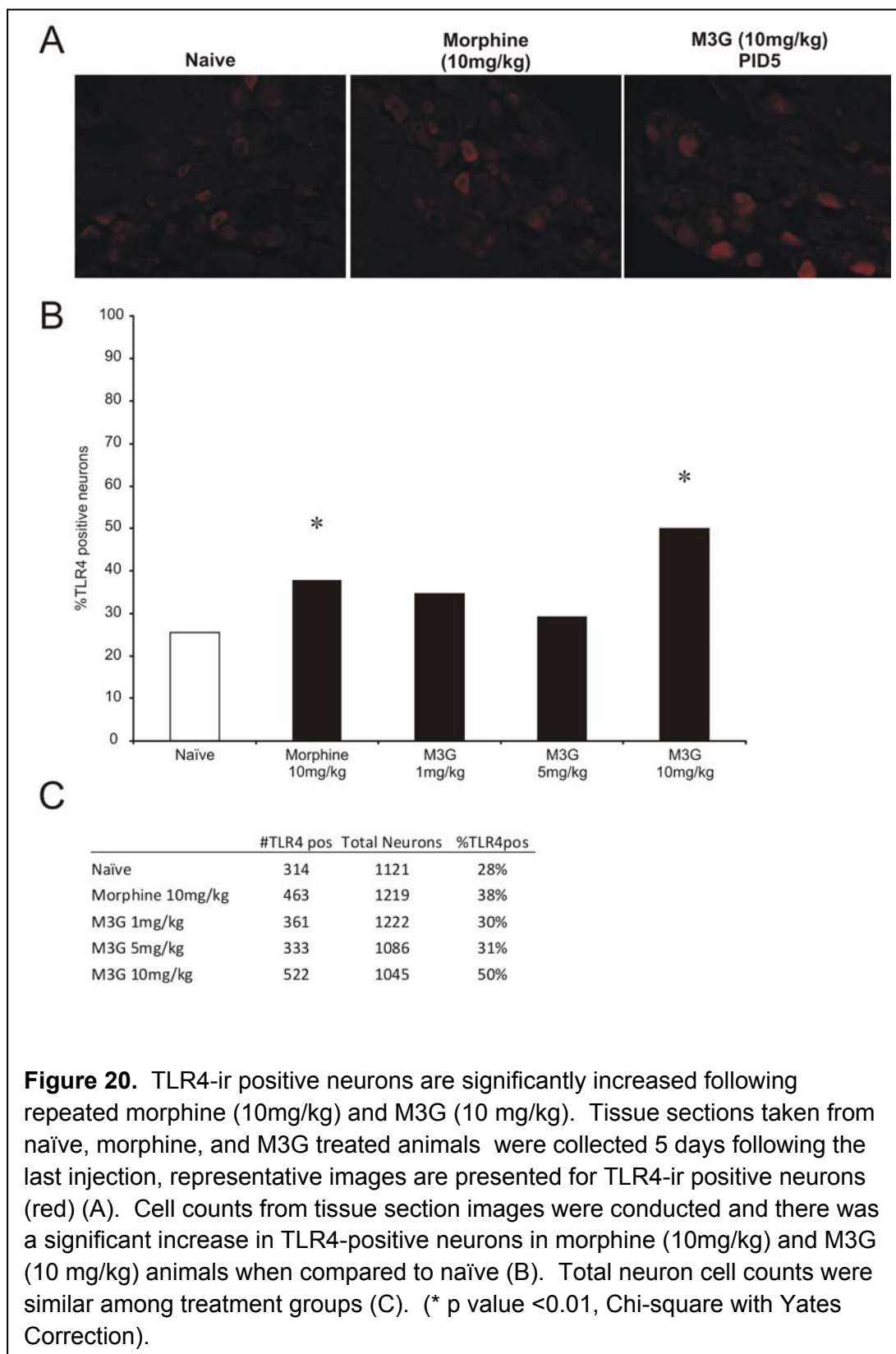
Repeated morphine and M3G administration induces a significant increase in TLR4-positive neurons.

Animals were subjected to repeated administration of morphine (10 mg/kg, once daily for 5 days) and M3G (1, 5, and 10 mg/kg, once daily for 5 days) and lumbar DRG were collected from animals 5 days following the last injection. TLR4-positive neurons (red) were present under all conditions (**Fig. 20A**). A significant increase in TLR4-immunopositive neurons was present following repeated morphine (10 mg/kg) and M3G (10 mg/kg) administration as compared to naïve DRG tissue (**Fig. 20B**). Each treatment condition had similar numbers of total numbers that were analyzed (**Fig. 20C**).

LPS responsive neurons are significantly increased following repeated morphine and M3G administration.

Lipopolysaccharide (LPS) is a major part of the outer component of gram negative bacteria that is crucial for the virulence of the bacteria (Cryz et al., 1984). LPS-induced effects have been demonstrated to dependent on binding to CD14 and TLR4 receptors (Pugin et al., 1993; Hoshino et al., 1999). Furthermore, LPS induced calcium response can be blocked by both CD14 and TLR4 antibodies (Song et al., 2001). Therefore, to investigate the presence of functional TLR4 receptors within the rodent DRG we applied LPS to acutely dissociated DRGs. The acutely dissociated DRG preparations were categorized into three neuronal and non-neuronal cell types: non-capsaicin sensitive neurons

(high K and ATP responsive), capsaicin sensitive neurons (capsaicin, high K, and ATP responsive), and glia (ATP responsive only). These cell response criteria were chosen strictly as an indicator of the types of cells that may be affected by the repeated morphine and M3G treatment paradigm. Naïve acutely dissociated DRGs produced LPS-induced $[Ca^{2+}]_i$ changes in a small percentage of neuronal and non-neuronal populations (**Table 4**). Repeated morphine (10 mg/kg) and M3G (10 mg/kg) treatment induced a significant increase in LPS calcium responsiveness of non-capsaicin sensitive and capsaicin sensitive neurons (**Table 4**). While the calcium imaging data does not conclusively demonstrate that increased TLR4 neuronal expression is causing the increased LPS responsiveness, it does demonstrate increased LPS response which is known to cause increase intracellular calcium levels through TLR4 (Song et al., 2001). This along with the increased TLR-4 positive neurons suggests TLR4 could be mediating this response (**Fig. 20B**). Further studies would need to be conducted to validate TLR4 is responsible for this increased signaling.



	Naïve	Morphine (10 mg/kg) Treated	M3G (5 mg/kg) Treated
	<i>Percentage Positive Response</i>		
Non-capsaicin sensitive neuron	6% (5/85)	23% (26/112)**	31% (23/74)**
Capsaicin Sensitive neuron	8% (6/72)	36% (26/73)**	25% (29/117)*
Glia	7% (3/44)	16% (8/49)	0% (0/25)

Table 4. Repeated morphine and M3G treatment significantly increase the percentage of LPS responsive neurons as measured by a change in intracellular calcium. Daily morphine and M3G administration (10 mg/kg for 5 days) were administered to animals. Lumbar DRG were acutely dissociated at 4-6 days following the last injection of morphine or M3G. (** p<0.001, * p<0.05, Chi-square with Yates correction).

APPENDIX B:
CCR2 RECEPTOR ANTAGONISM DOES NOT REVERSE MORPHINE-
INDUCED TACTILE HYPERNOCEPTION

Methods

Animals. Pathogen-free, adult female Sprague-Dawley rats (150–200 g; Harlan Laboratories, Madison, WI) were housed in temperature ($23 \pm 3^{\circ}\text{C}$) and light (12-h light: 12-h dark cycle; lights on at 07:00 h) controlled rooms with standard rodent chow and water available *ad libitum*. Experiments were performed during the light cycle. These experiments were approved by the Institutional Animal Care and Use Committee of Loyola University, Chicago. All procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health and the ethical guidelines of the International Association for the Study of Pain. All animals were randomly assigned to either treatment or control groups.

Drugs and method of administration The drugs, morphine sulfate salt and the CCR2 antagonist (R)-4-Acetyl-1-(4-chloro-2-fluorophenyl)-5-cyclohexyl-3-hydroxy-1,5-dihydro-2H-pyrrol-2-one (CCR2 RA), were employed in this study. Morphine sulfate salt and CCR2 RA were supplied by the NIDA Drug Supply Program (Rockville, MD) and Eli Lilly (Indianapolis, IN), respectively. All drugs were freshly prepared in saline on the day of the experiment. Morphine sulfate- and vehicle-treated groups were given intraperitoneal (i.p.) injections once daily for 5 days of 10 mg/kg or saline (vehicle). After tactile hypernociception was established, animals were given an i.p. injection of CCR2 RA (10 mg/kg) 5 days following the last morphine injection (PID5).

Tactile Behavioral assessment von Frey filaments were used to test mechanical sensitivity before, during and after cessation of morphine sulfate administration. Prior to initial von Frey tactile testing, all rodents were habituated to testing chambers for at least two days. Animals were tested for baseline responses (BL) at least two times before undergoing the repeated morphine sulfate treatment (10 mg/kg, i.p. daily). Mechanical testing with von Frey filaments during the morphine sulfate dosing paradigm was limited to injection day (ID) 3. Behavioral assessment on ID3 occurred 18-20 hours after the ID2 morphine administration and before ID3 morphine or vehicle treatment. Additional behavioral assessment following drug or vehicle administration occurred on post-injection day (PID) 3, 5, 7, and 14. All behavioral testing was performed by laboratory assistants who were blinded to the experimental conditions and unfamiliar with the experimental aims.

The incidence of foot withdrawal in response to mechanical indentation of the plantar surface of each hindpaw was measured with a von Frey filament capable of exerting forces of 10, 20, 40, 60, 80 and 120 mN. These probes exhibit a uniform tip diameter (0.2 mm) and were applied to 6 designated loci distributed over the plantar surface of the foot (Ma et al., 2003). These 6 spots are representative of the distal nerve distributions of saphenous, tibial and sural nerves (medial to lateral) in the glabrous hindpaw. During each test, the rodent was placed in a transparent plastic cage with a floor of wire with $\sim 1 \times 1$ cm openings. The cage is elevated so that stimulation can be applied to each hind

foot from beneath the rodent. The filaments were applied in order of ascending force. Each filament was applied alternately to each foot and to each locus. The duration of each stimulus was approximately 1 s and the inter-stimulus interval was approximately 10–15 s. The incidence of foot withdrawal is expressed as a percentage of the 6 applications of each stimulus and the percentage of withdrawals was then plotted as a function of force (Bhangoo et al., 2007a; Ma et al., 2003). The von Frey withdrawal threshold was defined as the force that evoked a minimum detectable withdrawal observed on 50% of the tests given at the same force level. For cases in which none of the specific filaments used evoked withdrawals on exactly 50% of the tests, linear interpolation was used to define the threshold.

Statistics. Prism 5 (GraphPad, LaJolla, CA) was used to determine the statistical significance of differences in the mean threshold forces for foot withdrawal to punctate indentation as a function of time and between experimental groups by means of one way analyses of variance (one-way ANOVA) followed by post hoc pairwise comparisons (Tukey method).

Results

Morphine-induced tactile hypernociception is not reversed with CCR2 receptor antagonist.

Animals were administered morphine (10 mg/kg, one a day for five days) and tactile behavior was assessed with von Frey filaments. Behavioral assessment of tactile hypernociception was performed prior to the start of the injection

paradigm (BL), during the 5 day dosing regimen (ID), and following the repeated morphine treatment paradigm (PID) (**Fig.21**). The mean paw withdrawal threshold (PWT) of the tested hind paws exhibited a significant decrease at ID4, PID3, and PID5 as compared to BL (**Fig. 21**). Administration of the CCR2 receptor antagonist (10 mg/kg, i.p.) at PID5 did not induce a significant change in PWT as compared to PID5 prior to injection (**Fig. 21**).

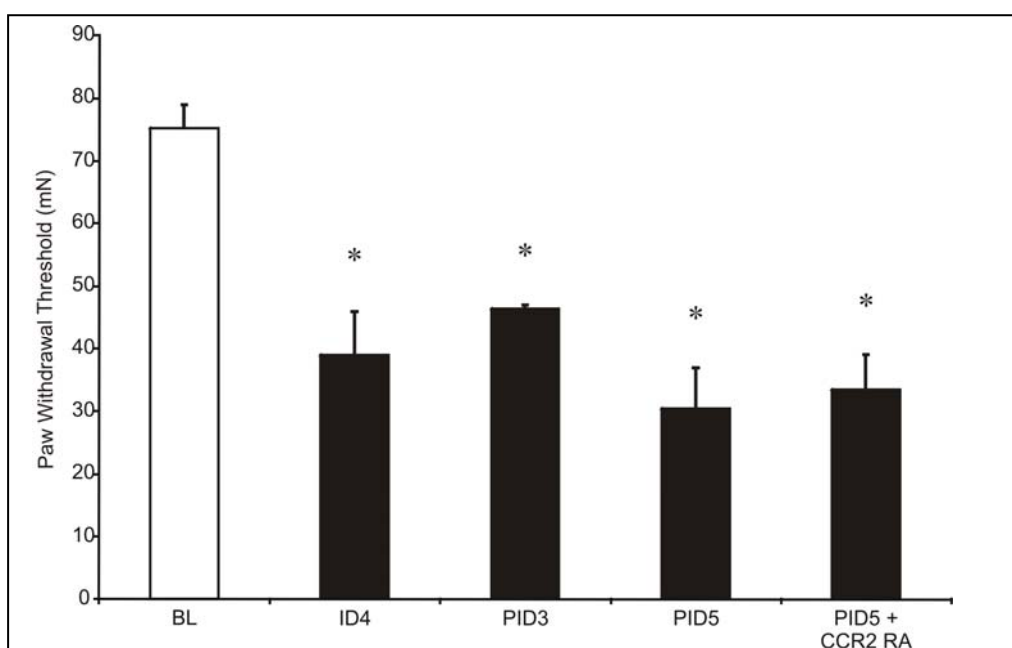


Figure 21. CCR2 receptor antagonism does not reverse morphine induced tactile hypernociception. Repeated morphine treatment (10 mg/kg for 5 days) results in the development of tactile hypernociception as measured by von Frey filaments. Five days following the last morphine injection (PID5) rats received an i.p. injection of CCR2 antagonist (10 mg/kg) and tactile behavior was measured by von Frey filaments 1 hour post injection. BL, baseline, ID, injection day, PID, post injection day, CCR2 RA, CCR2 receptor antagonist (One-way ANOVA; * $p < 0.05$, significant difference from baseline) ($n=6$)

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VITA

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While at Loyola, Natalie served on several committees, including the Pharmacology Department Student Representative to Faculty, Graduate Student Advisory Committee, and Graduate Student Committee. Natalie also received an NSF GK-12 Fellowship from June 2010 through May 2011.

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Date

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